Is flaxseed equivalent and/or synergistic with ACE inhibition in the treatment of chemotherapy induced cardiotoxicity?

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

Master of Science

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Abstract

Background: Breast cancer is a major public health concern in Canada. Although the current combination of surgery, radiation, and chemotherapy may lead to a cure in the breast cancer setting, the administration of the anti-cancer drugs Doxorubicin and Trastuzumab (DOX+TRZ) is associated with an increased risk of developing heart failure. Little is known about whether flaxseed (FLX) is equivalent to, or synergistic with, angiotensin converting enzyme inhibition (ACEi) in the treatment of DOX+TRZ mediated cardiotoxicity.

Objective: The specific aim is to evaluate whether FLX is comparable and/or incremental to standard pharmacological therapy using the ACEi Perindopril (PER) in the treatment of DOX+TRZ mediated cardiotoxicity.

Methods: In a chronic *in vivo* murine model of chemotherapy mediated cardiotoxicity, DOX+TRZ (8mg/kg and 3mg/kg, respectively) were administered weekly for a total of 3 weeks. Following this regimen, the mice were randomized to daily consumption of a 10% FLX supplemented diet, administration of PER (3mg/kg) *via* oral gavage, or a combination of both FLX+PER for an additional 3 weeks. Serial echocardiography was performed weekly. At the end of week 6, the mice were euthanized, and histological and biochemical analyses were performed on cardiac tissue and plasma.

Results: In mice treated with DOX+TRZ, the left ventricular ejection fraction (LVEF) decreased from 74±4% at baseline to 39±5% at week 6. Treatment with either FLX, PER, or FLX+PER improved LVEF to 63±4%, 64±3%, and 65±4%, respectively ($p<0.05$). Histological analyses

confirmed significant disruption of myofibrils, vacuolization, and loss of sarcomere integrity in the DOX+TRZ treated mice. Treatment with FLX, PER, or FLX+PER, however, improved myofibril integrity at week 6 in mice receiving DOX+TRZ. Although Bcl-2 interacting protein 3 (Bnip-3) and high mobility group box 1 protein (HMGB1) expression were significantly elevated in mice receiving DOX+TRZ, these markers of mitochondrial damage and cell death were attenuated by treatment with either FLX, PER, or FLX+PER. Finally, oxylipin analysis showed that 18-hydroxy-eicosatetraenoic acid (18-HETE) and 20-carboxy-arachidonic acid (20-COOH-AA) concentrations were significantly elevated in mice receiving DOX+TRZ; increases in concentrations of these oxylipins were attenuated by treatment with either FLX, PER, or FLX+PER.

Conclusion: In a chronic *in vivo* murine model of DOX+TRZ induced cardiotoxicity, FLX was equivalent to PER at improving cardiovascular remodeling, reducing histopathological changes in cardiomyocyte ultrastructure, and reducing biomarkers of inflammation, mitochondrial damage, and cell death in the treatment of cardiotoxicity, but the combination of FLX+PER was not synergistic.

Acknowledgements

My deepest gratitude goes first to Dr. Davinder Jassal, whose mentorship and high expectations pushed me to accomplishments beyond what I ever anticipated of myself. His guidance, enthusiasm, and patience reach far beyond the realm of research, always demonstrating a sincere desire to see his students succeed and unwavering faith in our potential. His role in shaping the confidence I have in my own abilities as a scientist will continue to act as a guiding force throughout my career. I would also like to extend my sincere gratitude to Dr. Pawan Singal for his wise and encouraging teaching throughout this project. His ability to open my eyes gently and swiftly to the breadth of knowledge that he had to share, and that I had yet to learn, proved invaluable throughout this project. He kept me searching for a deeper understanding of my research. To the rest of my advisory committee: Dr. Ian Dixon, Dr. Amir Ravandi, and Dr. Jeff Wigle, I am eternally grateful for their support and expertise throughout this project to advance my knowledge and refine this study.

Words cannot express the debt of gratitude I owe to Mr. David Cheung. His constant support every step of the way, and his patience teaching me all the necessary lab skills, are what made this project possible. He moves seamlessly between the roles of mentor, colleague, and friend taking pride in both his work and mine. His dedication to this project remains unmatched, and it has been a true honour to work alongside him. I would also like to express my appreciation to my other colleagues in the Cardiovascular Imaging Laboratory including: Vibhuti Arya, Paris Haasbeek, Ashley Bryson, and Lana Mackic. Their contributions to the supportive and entertaining atmosphere of the lab made every day truly enjoyable. I am proud to say that I have not only gained them as co-workers, but also as friends, and I look forward to all that they will accomplish in the future.

I would also like to acknowledge many staff members at the Saint Boniface Research Centre and the University of Manitoba for their contributions to this project. I would like to thank Rob Mazur, Nancy Lemoine, Dana Thompson, Sheri Bage, Nicole Whyte, Noémie Durand, and the rest of the staff at the Burrell Animal Holding facility for their skillful and caring support. I extend a sincere thank-you to the technicians in the Institute of Cardiovascular Sciences, particularly Teri Moffatt, Alex Austria, Matthew Guberman, Kim O'Hara, Allison Ledingham, Robert Fandrich, and Tanja Winter, for their willingness to answer questions and assist with lab work. They truly go above and beyond, and their dedication to the students does not go unnoticed. I would like to thank Cameron Eekhoudt, Sikta Chattopadhyaya, and Akshi Malik for their guidance in navigating the victories and challenges of my graduate program. I would also like to extend a thank-you to Dr. James Thliveris for contributing his expertise in histology and electron microscopy to advance this project, and to Dr. Harold Aukema for taking the time to walk me through the world of oxylipins.

Lastly, I would like to thank my family, my parents Sharlene and David, as well as Josh, Anna, and Neill for their unconditional love and support. The sacrifices they have made to stay by my side through every late-night study session and stressful exam are an immeasurable contribution to my education. They have always believed in me even when I have struggled to believe in myself, and I am eternally grateful to have them in my life. This achievement would truly not have been possible without each and every one of you.

In dedication to

John Richard Douglas Stevenson

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List of Abbreviations

20-COOH-AA	20-carboxy-arachidonic acid
AC	Adriamycin and Cyclophosphamide
ACEi	Angiotensin converting enzyme inhibitor
ALA	Alpha-linoleic acid
ANG-II	Angiotensin-II
ANOVA	Analysis of variance
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma extra-large
Bcl-XS	B-cell lymphoma extra-small
Bnip-3	Bcl-2 interacting protein 3
BNP	Brain-type natriuretic peptide
BP	Blood pressure
BRCA	Breast cancer tumor suppressor genes
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BSA	Bovine serum albumin
β-blocker	Beta-adrenergic receptor blocker
CMR	Cardiac magnetic resonance
COX	Cyclooxygenase

CRP	C-reactive protein
cTnT	Cardiac troponin-T
CTRCD	Cancer therapy related cardiac dysfunction
CYP	Cytochrome P450
DBP	Diastolic blood pressure
DCL	Docetaxel
DOX	Doxorubicin
DRI	Direct renin inhibitor
EBC	Early-stage breast cancer
ECL	Enhanced chemiluminescence
EpDPE	Epoxy-docosapentaenoic acid
EpETE	Eicosatetraenoic acid
EpETrE	Epoxy-eicosatrienoic acid
EPR	Epirubicin
ER	Estrogen receptor
FEC	5-Flurouracil, Epirubicin, and Cyclophosphamide
FLX	Flaxseed
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLS	Global longitudinal strain
HDoHE	Hydroxy-docosahexaenoic acid
HEPE	Hydroxy-eicosapentanoic acid
HER2	Human epidermal growth factor receptor 2
HMGB1	High mobility group box 1 protein

HR	Heart rate
HETE	Hydroxy-eicosatetraenoic acid
HPLC	High performance liquid chromatography
IL-1β	Interleukin 1-beta
IL-6	Interleukin-6
INF-α	Interferon-alpha
i.p.	Intraperitoneal
IVS	Interventricular septal wall thickness
LOX	lipoxygenase
LV	Left ventricle/ventricular
LVEDD	Left ventricular end-diastolic diameter
LVEF	Left ventricular ejection fraction
LVESD	Left ventricular end-systolic diameter
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MI	Myocardial infarction
MRA	Mineralocorticoid receptor antagonist
MUGA	Multiple-gated radionuclide angiography
NF-κβ	Nuclear factor kappa beta
NT-pro-BNP	N-terminal pro-brain type natriuretic peptide
PARP	Poly-ADP-ribose polymerase
PCL	Paclitaxel

PER	Perindopril
PLAX	Parasternal long axis
PP	Pulse pressure
PPAR	Peroxisome proliferator-activated receptor
PSAX	Parasternal short axis
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
PWT	Posterior wall thickness
RAS	Renin-angiotensin system
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SDG	Secoisolariciresinol diglucoside
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SMP	Skim milk powder
TDI	Tissue doppler imaging
TnI	Troponin I
TNF-α	Tumor necrosis factor alpha
TRZ	Trastuzumab
TTE	Transthoracic echocardiography

Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Epidemiology

According to the Canadian Cancer Society, breast cancer is the most common type of cancer with the second highest mortality rate for Canadian women.[1] It is estimated that in 2022, more than 110,000 Canadian women will receive a cancer diagnosis, and 28,600 of those diagnoses will be breast cancer.[1] While the breast cancer death rate has been declining since 1986, and the 5-year survival rate is estimated at 89%, more than 5,500 Canadian will die from breast cancer in 2022, representing 14% of all cancer deaths.[1] It is estimated that 1 in 8 Canadian women will be diagnosed with breast cancer, and 1 in 34 will die from the disease.[1] Breast cancer affects women with a wide age distribution, with nearly 40% of diagnoses in females between 30 and 59 years of age. Cancer-related healthcare costs continue to rise, with breast cancer as a leading contributor, creating an immense economic burden. The rising global disease burden exemplifies the need for further scientific exploration to improve breast cancer treatment, despite the improved survival rate for breast cancer patients.[2]

1.1.2 Risk Factors

While the overall incidence of breast cancer is very high, there are well established risk factors that increase an individual's risk of developing the disease.[3] These risk factors can be categorized into biological risk factors and lifestyle/socioeconomic risk factors, with biological factors posing the most pertinent risk.[3-7] Mutations in the breast cancer tumor suppressor genes (BRCA), specifically the breast and ovarian susceptibility genes breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2), are the greatest contributor resulting in more than an 80% increased

lifetime risk of developing breast cancer.[5-8] Family history is the second greatest risk factor with a positive first-degree family history doubling an individual's risk of developing breast cancer.[1] Other heritable risk factors for breast cancer include atypical hyperplasia, increased breast density, age, and/or late menarche.[3] Lifestyle and socioeconomic factors that increase the risk of breast cancer development include previous chest wall irradiation, hormone replacement therapy, lack of physical activity, excess adipose tissue, smoking, and high cumulative alcohol intake.[4,6,9-11]

1.1.3 Diagnosis

Breast cancer mortality rates have decreased by nearly 50% since 1986, and the decline can partially be attributed to increased screening allowing for earlier detection and treatment.[1] The Canadian Cancer Society has established screening mammography guidelines suggesting optional screening for women between 40 and 49 years of age with identified risk factors, and biennial screening for women between the ages of 50 and 74.[1] The World Health Organization suggests that breast cancer mortality could be decreased by approximately 20% globally through the use of similar screening programs.[3] While mammography is an effective non-invasive screening tool, conclusive diagnosis of breast cancer requires microscopic analysis of core needle biopsies to determine tumor grade, cancer stage, and aggressiveness of the neoplasm. These insights are then used in the development of the multimodal treatment plan likely to be most effective.

1.1.4 Treatment

Cancer treatments continue to become more effective through global research efforts, and early diagnosis often allows for life-saving treatment. Breast cancer treatment typically employs a combinatorial approach of surgery, radiation, chemotherapy, and/or targeted biological therapy.

Surgical interventions for breast cancer treatment were the first major step in improving patient survival. Surgical options include mastectomies and lumpectomies. Lumpectomies have become the preferred surgical method due to the less invasive breast conserving nature of the approach.[12,13] However, this approach can only be used if the breast tumor and a surrounding margin of healthy tissue can be removed, so mastectomies are still required in some cases. Since surgery alone does not prevent breast cancer recurrence, the treatment approach typically includes radiation and chemotherapy to improve local control, distant control, and overall survival.[14]

Radiation therapy is most commonly used as an adjuvant treatment following surgical intervention and/or chemotherapy.[1] This treatment approach reduces the rate of recurrence following a lumpectomy by 50% and increases the breast cancer survival rate by approximately 17%.[15] Radiation therapy can also be used prior to surgery as a means to reduce the size of the tumor, facilitating surgical resection. Neoadjuvant radiotherapy has been shown to improve pathologic response, decrease the rate of recurrence, and increase overall survival compared to adjuvant radiotherapy.[16,17]

The most common treatment for early-stage breast cancer (EBC) is chemotherapy. While there are a variety of effective chemotherapy regimens, their usage and efficacy vary based on the stage of breast cancer.[18] For EBC, the most frequently prescribed are third generation regimens, which include a combination of taxanes and anthracyclines as anti-cancer agents. The taxane class includes Paclitaxel (PCL) and Docetaxel (DCL), and the anthracycline class incudes Doxorubicin (DOX) and Epirubicin (EPR).[18,19] While these regimens are effective treatments in the breast

cancer setting, the use of anthracyclines is associated with a 10% risk of developing cardiotoxicity that is dose dependent.[20,21]

Targeted biological therapies are the latest approach to cancer treatment, and their use varies based on the specific characteristics of the breast cancer. One key factor in determining breast cancer progression is the hormone receptor status, which indicates the level of neoplastic cell dependency on hormones for survival and proliferation.[22] Approximately 75% of breast cancers are estrogen receptor (ER) positive and approximately 20% are human epidermal growth factor receptor 2 (HER2) positive. While positivity for either receptor worsens patient prognosis, it also provides focused therapeutic opportunities.

ER positive breast cancer is treated using pharmaceuticals that reduce estrogen signaling in the neoplastic environment in order to limit cell survival and proliferation. Selective estrogen receptor modulators (SERMs) bind to the ER in order to inhibit estrogen uptake.[23] Estrogen-receptor down-regulators induce degradation of the ER to inhibit estrogen binding. Luteinizing hormone-releasing hormone agents are effective in pre-menopausal women by inhibiting ovarian production of estrogen. Finally, aromatase inhibitors are effective in post-menopausal women by inhibiting the conversion of androgens to estrogen.[24] These four mechanistically independent treatments result in a similar decrease in estrogen bioavailability.

HER2 positive breast cancer presents with increased receptors that promote the growth and proliferation of cancer cells, making HER2 positive breast cancer more aggressive. Trastuzumab (TRZ) is a monoclonal antibody that specifically targets the HER2 protein to block its promotive

action of cancer development. TRZ is the most commonly used monoclonal antibody in both the adjuvant and metastatic breast cancer settings to improve patient survival and reduce risk of recurrence, and is a standard component in the treatment of HER2 positive breast cancer.[25-28]

In the adjuvant setting, TRZ is administered following the completion of anthracycline-based chemotherapy as a loading dose of 8mg/kg then maintenance doses of 6mg/kg every three weeks for a total of 18 cycles.[29] Several studies have validated the use of TRZ in combination with anthracycline based chemotherapies.[25,27,29-39] The role of TRZ in the clinical setting has been evaluated by a number of multi-centred randomized clinical trials in which patients received a cumulative dose of DOX or EPR of 360mg/m^2 and 720mg/m^2, respectively, followed by one year of adjuvant treatment with TRZ. These trials demonstrated that adjuvant use of TRZ can reduce the overall rate of recurrence by 50% and cancer mortality by 33% in women expressing HER2 positive breast cancer.[31-38]

In the metastatic setting, TRZ is administered as a loading dose of 4mg/kg then maintenance doses of 2mg/kg every three weeks for a duration of one year.[40] In the breast cancer population, 10% of patients develop metastatic malignancies, and 35-45% of metastatic breast cancer patients are HER2 positive.[25] Although palliative, chemotherapeutic agents have been shown to improve the short term survival of metastatic breast cancer patients, and the recent addition of TRZ for HER2 positive metastatic breast cancers has significantly improved the survival outcome in this patient population.[25,41,42] Monotherapy with TRZ demonstrated a 26% response rate in a clinical trial of metastatic HER2 positive breast cancer patients without prior use of an anthracycline based chemotherapy.[43] When TRZ is used in conjunction with anthracyclines in women with HER2

positive metastatic breast cancer, mortality risk is reduced by 20%.[44] While TRZ is an effective anti-cancer agent, it also presents with cardiotoxic side effects.[21,25,45]

1.2 Cardio-Oncology

Cardio-Oncology is a novel field of research focused on the prevention, diagnosis, management, and treatment of chemotherapy induced cardiotoxicity. It has emerged due to the increasing prevalence of patients developing heart failure, or other cardiovascular disease, following chemotherapy treatment. Although the current combination of surgery, radiation, chemotherapy, and targeted therapies are effective treatments in the breast cancer setting, their clinical use is limited due to the cardiotoxic side effects. Cardiac dysfunction is the leading cause of morbidity and mortality among cancer patients, despite significant advances in oncology over the last few decades.[13] In order to standardize the diagnostic criteria for cancer therapy related cardiac dysfunction (CTRCD), the American Society of Echocardiography developed the defining parameters of a >10% decrease in left ventricular ejection fraction (LVEF) compared to a baseline value or an absolute LVEF of <53% following chemotherapy treatment.[46] To help establish earlier detection of CTRCD, a >15% decrease in global longitudinal strain (GLS) may be used as an earlier predictor of CTRCD than LVEF, despite being considered subclinical left ventricular (LV) systolic dysfunction.[46] There is also ongoing research investigating the utility of biomarkers of myocardial damage, such as cardiac troponins and brain-type natriuretic peptide (BNP), for early detection and risk stratification of CTRCD.[47] Typically, upon detection of CTRCD, the offending anti-cancer agents are withheld and patients are prescribed low-doses of an angiotensin converting enzyme inhibitor (ACEi) and a beta-adrenergic receptor blocker (β-blocker) to reverse the existing damage and prevent further cardiac deterioration.[48]

1.2.1 Chemotherapy & Targeted Biological Therapy

Anthracyclines, such as DOX and EPR, are the primary culprit in the development of chemotherapy mediated cardiotoxicity. These chemotherapies cause dose-dependent type-I cardiotoxicity in approximately 10% of patients. In type-I cardiotoxicity, anthracyclines induce necrosis and apoptosis of cardiac myocytes followed by permanent myocardial fibrosis (Table 1).[21,46] This heart damage limits the maximum dose of anthracyclines that can be safely administered as an anti-cancer agent.[21]

The known cardiotoxic mechanisms of DOX include inflammation and oxidative stress induced cell damage and/or death that lead to adverse cardiac remodelling. Inflammation is the result of a complex biological response that is intended to protect the body from harmful stimuli including pathogens, infections, and/or tissue damage. Inflammation involves the recruitment of immune cells, blood vessels, as well as several molecular mediators, which eliminate the initial cause of cell injury, necrotic cells, and/or damaged tissue, and initiate the repair process.[49-51] Nuclear factor kappa beta (NF-κβ) is a molecular mediator that acts as a regulator of the acute phase of inflammation that up-regulates downstream pro-inflammatory biomarkers including tumor necrosis factor alpha (TNF-α), interferon-alpha (INF-α), interleukin 1-beta (IL-1β), and interleukin-6 (IL-6), which can lead to cardiac fibrosis and heart failure.[49-53] NF-κβ can also be activated by TNF-α and IL-1β in a feedback loop.[52] In addition to directly causing inflammation, DOX causes upregulation of other bioactive molecules, such as proteins and oxylipins, that can themselves increase inflammatory processes. The administration of anthracycline based chemotherapeutic agents, such as DOX, is associated with increased inflammation within the myocardium and vasculature that can lead to long-term damage.[50]

Oxidative stress results from an accumulation of reactive oxygen species (ROS) beyond the biological system's ability to neutralize these reactive intermediates. ROS are produced by the incomplete reduction of oxygen which results in singlet oxygen molecules, superoxide radicals, and hydroxide radicals that are detrimental to surrounding tissues as they have short half-lives, an unstable nature, and are highly reactive.[54,55] An end product of oxidative stress is cardiomyocyte dysfunction induced by the auto-oxidation of catecholamines or the interference of ROS with the calcium transport in the sarcoplasmic reticulum.[49,54]

Anthracyclines are known to cause oxidative stress by up-regulating the mitogen-activated protein kinase (MAPK) and the peroxisome proliferator-activated receptor (PPAR) signaling pathways.[56-58] The MAPK cascade relays intracellular signals from the cell surface to the nucleus that directly influence the genetically programmed cell death pathway known as apoptosis.[58] In response to anthracyclines, this pathway increases expression levels of pro-apoptotic proteins including Bcl-2 associated X protein (Bax), Caspase-3, and poly-ADP-ribose polymerase (PARP), while decreasing expression levels of anti-apoptotic proteins including B-cell lymphoma extra-large (Bcl-XL).[59-63] PPARs are ligand-activated transcription factors involved in inflammation, fatty acid β-oxidation, and cholesterol metabolism.[56,64-68] PPAR-α is abundantly expressed in heart tissue, which utilizes fatty acid β-oxidation as the major source of energy.[56,67,69-72] When the toxic effects of anthracyclines place an increased energy demand on the heart, higher levels of oxygen are consumed causing an imbalance in the production and neutralization of ROS.[56,67,71,72] To compensate for the increase in fatty acid β-oxidation, PPAR-α protein expression levels are increased, which predisposes the heart to contractile dysfunction, eventually resulting in DOX mediated cardiomyopathy.[57]

The oxidative stress occurring in the cardiomyocytes can also cause mitochondrial damage, as indicated by increased expression levels of Bcl-2 interacting protein 3 (Bnip-3), which further contributes to the energy crisis in cells with increased energy demand.[73] While these oxidative stress induced pathways often trigger apoptosis, they can also induce other cell death pathways including necrosis and ferroptosis, as indicated by increased expression levels of high mobility group box 1 protein (HMGB1). The multifactorial nature of DOX+TRZ mediated cardiotoxicity, including the up-regulation of inflammatory pathways and increased ROS production, presents a challenge surrounding the mitigation of this adverse side-effect. This study will aim to elucidate the mechanistic roles of inflammation and oxidative stress in DOX+TRZ mediated cardiotoxicity and investigate whether the anti-inflammatory and antioxidant agent flaxseed (FLX) may treat this adverse side effect.

The two most common currently used chemotherapeutic regimens in the treatment of breast cancer are 5-Flurouracil, EPR, and Cyclophosphamide (FEC) and Adriamycin and Cyclophosphamide (AC), both of which contain anthracyclines. In the breast cancer setting, FEC chemotherapy is typically administered as an intravenous infusion once every three weeks, for four to six consecutive cycles, up to cumulative doses of 500mg/m^2 5-Flurouracil, 100mg/m^2 EPR, and 500mg/m^2 Cyclophosphamide or until contraindications emerge. AC is typically administered once every two weeks as an intravenous infusion of 60mg/m^2 Adriamycin and 600mg/m^2 Cyclophosphamide for three consecutive cycles either before or after surgical resection of the tumor. If there is evidence of disease progression, or the development of cardiac dysfunction, AC chemotherapy is stopped.

Clinically, DOX is used as a chemotherapeutic agent to treat both solid and hematologic primary cancers and malignancies, including breast cancer.[74-76] This treatment, however, is limited by its cumulative dose-dependent side effect of cardiotoxicity. Since DOX remains a primary cancer therapy despite these side effects, considerable effort needs to be made to prevent, detect, and treat cardiac function declines in this patient population.

Despite the beneficial effects of the monoclonal antibody TRZ, which is used as a targeted biological therapy against HER2 positive breast cancers, it is also associated with an increased risk of cardiotoxicity. The cardiac dysfunction caused by this anti-cancer agent manifest in two ways. Alone, TRZ causes type-II cardiotoxicity resulting in dilated cardiomyopathy marked by a reduction in LVEF (Table 1). However, the cardiotoxic effects are typically reversible with temporary discontinuation of the therapy.[74,77] More devastatingly, when used in conjunction with anthracyclines, TRZ potentiates the type-I cardiotoxicity, elevating the risk of developing permanent heart damage up to 25%.[78,79] This further limits the maximum dose of chemotherapy administered in the breast cancer setting.[80,81] Despite their prolongation of the lives of many breast cancer patients, the cardiovascular side effects notably diminish the discernible benefits of these anti-cancer therapies.

The cardiotoxic mechanisms of TRZ occur primarily through increased apoptosis and oxidative stress. The binding of TRZ to HER2 receptors in cardiomyocytes alters the expression levels of pro- and anti-apoptotic protein levels, specifically the B-cell lymphoma 2 (Bcl-2) family of proteins containing pro-apoptotic Bax and B-cell lymphoma extra-small (Bcl-XS) and anti-apoptotic Bcl-XL.[82,83] As pro-apoptotic expression increases and anti-apoptotic expression

decreases, the shift in protein ratios activates apoptotic cell death.[82] Oxidative stress induced cardiomyocyte damage occurs due to upregulation of the renin-angiotensin system (RAS) and the anti-dimerization properties of TRZ, both of which are associated with the downregulation of cell survival pathways.[84,85] When TRZ binds to the HER2 receptors, it inhibits cell survival pathways, thus stressing the myocardium.[86] This stress significantly increases circulating levels of angiotensin-II (ANG-II), which can then bind the angiotensin-1 receptor, activating a cascade that produces ROS, leading to oxidative stress.[54,87-91] This ANG-II induced oxidative stress also leads to increased expression levels of pro-apoptotic genes, including Bax, Caspase-3, and PARP, and decreased expression levels of anti-apoptotic genes, including Bcl-XL.[59-63,92,93] The binding of TRZ to a HER2 receptor prevents dimerization to another HER family receptor, which downregulates cell survival pathways, making the cardiomyocytes susceptible to damage.[85,94-100] Due to the high metabolic demand for adenosine triphosphate (ATP), active cardiomyocytes have increased activity of the electron transport chain in the mitochondria, which potentiates the production of ROS.[87,97] With the co-occurrence of damage susceptibility and the presence of ROS, cardiomyocytes are unable to compensate for the oxidative stress, which initiates apoptosis, resulting in cardiac dysfunction.[97,100-102]

Feature	Type I Cardiotoxicity	Type II Cardiotoxicity
Anti-Cancer Agent	Anthracyclines (DOX, EPR)	Trastuzumab
Dose Dependency	Yes	No
Anti-Cancer Mechanism	Topoisomerase inhibition	Cell signalling interruption
Cardiotoxic Mechanism	Inflammation, Oxidative stress, Mitochondrial damage, Cardiomyocyte death	Oxidative stress, Cardiomyocyte damage
Reversibility	Irreversible	Possible functional recovery after discontinuation
Ultrastructural Change	Vacuolar swelling, Myofibrillar disarray, Cell death	May not lead to apoptosis in isolation
Rechallenge After Cardiotoxicity	Not safe	May not result in further cardiotoxicity
Risk Factors	Combinational chemotherapy, Neoadjuvant radiotherapy, Age, Previous cardiac disease, Hypertension	Neoadjuvant chemotherapy, Age, Previous cardiac disease, Obesity

Table 1. Comparison of the features of type I and type II cardiotoxicity.

1.2.2 Cancer Therapy Related Cardiac Dysfunction

Chemotherapy mediated cardiotoxicity typically manifests as congestive heart failure, but it can also present as ischemia, hypertension, pericarditis, and/or arrhythmias.[103,104] Following administration of DOX+TRZ, up to 25% of patients show increased biomarkers of inflammation, cardiac injury, and remodelling as well as reduced LVEF and GLS parameters.[105,106] A retrospective analysis of clinical trials found that the estimated percentage of patients who develop

DOX induced congestive heart failure ranges from 5% at a cumulative dose of 400mg/m^2 to 48% at a cumulative dose of 700mg/m^2, although the clinical cumulative dose of DOX is now limited to 500mg/m^2 to prevent such high risk.[76] A retrospective clinical study of DOX+TRZ found that up to 22% of patients required their anti-cancer treatment to be discontinued due to the development of LV systolic dysfunction, and that 40% of patients experiencing cardiotoxicity showed equivalent or worsening LVEF over time despite receiving optimal medical treatment.[79] As a major contributor to long-term morbidity and mortality among cancer survivors, chemotherapy mediated cardiotoxicity is defined as an absolute LVEF of <53% or a 10% drop in LVEF from the patient's baseline value.[107]

In cases where cardiotoxicity is discovered, repeat cardiac imaging should be performed 2 to 3 weeks after the initial diagnosis in order to corroborate initial findings, monitor progression, and deduce degrees of reversibility.[46] If the LVEF recovers to within 5 percentage points of the baseline value, the cardiotoxicity is considered reversible. If the LVEF recovers by ≥10 percentage points but remains more than 5 percentage points below the baseline value, the drug induced cardiotoxicity is considered partially reversible. If the LVEF recovers by <10 percentage points and remains more than 5 percentage points below the baseline value, the cardiotoxicity is considered irreversible.[46]

1.2.3 Cardiovascular Imaging

There are multiple cardiovascular imaging techniques that can be used to monitor changes in cardiac function. The primary imaging techniques used to diagnose and monitor cardiotoxicity in breast cancer patients include multiple-gated radionuclide angiography (MUGA), cardiac

magnetic resonance imaging (CMR), and transthoracic echocardiography (TTE). While the choice of imaging modality can vary based on resource availability, the imaging modality should remain consistent within the same patient over time to compare images and measure changes in cardiac function more accurately.

MUGA scans are a form of nuclear imaging used to measure LVEF. The scan involves injection of a radionucleotide tracer that is then imaged using a gamma camera to capture images of the heart at specific timepoints in the cardiac cycle. MUGA scans are used for the assessment of cardiac function in breast cancer patients because it is an accessible imaging modality with a high degree of reproducibility. These features allow for easy repeat imaging throughout cancer treatment and accurate assessment of changes in LVEF.[108] However, MUGA scans do not provide any information about cardiac structure (i.e. valves) and expose patients to radiation (~5-10 mSv/scan) thus limiting their efficacy.[109] Moreover, MUGA scans are limited due to their reliance on LVEF as the sole measure of cardiac function which is not sufficient for early detection of CTRCD.[110]

CMR is the current gold standard for assessment of ventricular volumes and ejection fraction due to its high image resolution and intra- and inter-observer reproducibility.[111] CMR is considered a suitable modality for early CTRCD detection due to its high tissue characterisation abilities and sensitivity to early changes in tissue composition including LV mass, inflammation, fibrosis, and myocardial strain.[112] However, the low availability and high cost of CMR present barriers that currently limit its feasibility in the context of Cardio-Oncology where repeat imaging is of paramount importance to follow patient progression.

TTE is the current standard of practice for cardiac assessment before initiation of chemotherapy and early detection of CTRCD. TTE is an advantageous imaging modality because it is widely available, versatile, easily repeatable, and does not expose the patient to radiation. TTE allows for characterization of both systolic and diastolic function of both ventricles as well as pulmonary pressures, valvular abnormalities, wall thicknesses, the pericardium, and GLS.[113] Three-dimensional TTE offers superior accuracy compared to 2-dimendional TTE decreasing the LVEF calculation error from 10% to less than 5%.[114] However, since three-dimensional TTE is not always available, the addition of contrast to 2-dimensional TTE can increase accuracy by improving definition of endocardial borders.[115]

While serial TTE, MUGA, and CMR are all non-invasive imaging techniques used to monitor for cardiac dysfunction through changes in LVEF, this is a late marker of myocardial injury. Recently, increased emphasis has been placed on novel echocardiographic techniques that are more effective for the early detection of chemotherapy mediated cardiotoxicity. Specifically, GLS, an echocardiographic measurement of myocardial strain analysis, used in conjunction with biomarkers of cardiac injury and inflammation, can detect early signs of chemotherapy mediated cardiotoxicity and is predictive of future reductions in LVEF.[21,109,116-118] A GLS greater than -17.5% (i.e. -17.0%) before initiation of anthracycline based chemotherapy increases the risk of developing CTRCD.[119] The development of CTRCD is indicated by a GLS change of >15% as compared to baseline in patients receiving cardiotoxic anti-cancer therapy.[107] GLS has been shown to be an effective predictor of DOX+TRZ mediated cardiotoxicity. In patients that eventually develop cardiotoxicity, increases in GLS values are evident as early as 3 months into treatment while changes in LVEF do not appear until 6 months into treatment, making GLS changes an

earlier echocardiographic marker of subclinical dysfunction prior to changes in LVEF.[106] Early detection is an important element to consider since current treatments are more effective if they are introduced sooner in the progression of cardiotoxicity. The most sensitive surveillance for the development of cardiotoxicity, thus allowing earlier initiation of cardioprotective interventions, is a combined approach involving serial imaging and monitoring of cardiac biomarkers.

1.2.4 Biomarkers

In the pre-clinical setting, there is a notable advantage for analysis of biomarkers due to the feasibility of directly analysing tissue samples instead of being primarily limited to blood samples. This allows for the assessment of cardiac proteins. In models of DOX+TRZ induced cardiotoxicity, an increased ratio between NF-κβ and its active form phospho-NF-κβ have indicated increased levels of inflammation.[120,121] As a marker of oxidative stress induced mitochondrial dysfunction, Bnip-3 expression in cardiomyocytes has been shown to increase due to DOX.[73] Other markers of oxidative stress that increase in DOX+TRZ mediated cardiotoxicity are the ratio between Bax and Bcl-XL, Caspase-3, and PARP all indicative of cardiomyocyte apoptosis.[60,122] Another biomarker that is elevated in DOX+TRZ induced cardiotoxicity is HMGB1, indicative of necrosis and ferroptosis (Table 2).[123-126]

Pathway	Protein
Oxidative Stress Induced Apoptosis	Bcl-2 associated X protein (Bax)
	B-cell lymphoma extra-large (Bcl-XL)
	Caspase-3
	Poly-ADP-ribose polymerase (PARP)
Inflammation	Nuclear factor kappa beta (NF-κβ)
	phospho- NF-κβ
Necrosis/Ferroptosis	High mobility group box 1 protein (HMGB1)
Oxidative Stress Induced Mitochondrial Dysfunction	Bcl-2 interacting protein 3 (Bnip-3)

Table 2. Protein markers of DOX+TRZ mediated cell damage pathways.

Apoptosis can be analyzed by quantifying both pro-apoptotic and anti-apoptotic protein expression. As part of its anti-cancer mechanism, DOX generates ROS to increase apoptosis in cancer cells.[122] While this is effective for reducing tumor size, the same oxidative stress induced apoptosis occurs in cardiomyocytes, contributing to cardiotoxicity.[60] Under normal conditions, the cell is in an anti-apoptotic state, meaning that the dominant expression is of anti-apoptotic proteins such as Bcl-XL. When the cell experiences oxidative stress, the ratio of pro-apoptotic to anti-apoptotic proteins shifts towards pro-apoptotic proteins such as Bax. While Bcl-XL is dominant, the cell continues to function, but once oxidative stress accumulates to a point where Bax becomes dominant, the shift in protein expression initiates apoptosis.[60,122] The apoptotic cascade activates Caspase-3 which then upregulates PARP expression (Figure 1).[60,122]

Inflammation can be analyzed by quantifying expression levels of pro-inflammatory proteins. NF-κβ is a protein complex that mediates DOX-related cardiac cell inflammation by controlling transcription of pro-inflammatory cytokines.[120,121] Elevations in both NF-κβ, and its active form phospho-NF-κβ (p65 subunit), are indicative of cardiac inflammation in response to DOX toxicity (Figure 1). In previous murine models of DOX induced cardiotoxicity, elevations in NF-κβ and phospho-NF-κβ have been observed in subjects receiving DOX alone, but the elevations have been attenuated by prophylactic administration of vitamin C, micheliolide, and other anti-inflammatory agents.[120,121] As the master regulator of DOX-induced inflammation, increased NF-κβ would also suggest the presence of increased IL-6, IL-1β, and TNF-α.[120,121]

DOX had been shown to activate both apoptotic and necrotic cell death pathways in cardiomyocytes.[120] HMGB1 is a signalling protein that is primarily a marker of necrosis, as it is passively released from necrotic cells. However, in pathological conditions, it can also trigger inflammation in neighbouring cells and may be actively secreted by viable cells. By interacting with its toll-like receptor-4, HMGB1 can activate NF-κβ to promote inflammation that can then contribute to DOX induced cardiomyocyte apoptosis and cell damage.[123] More recent studies suggest that the HMGB1 increase is associated with ferroptotic cell death. DOX induces mitochondrial damage, that causes iron accumulation within the mitochondria, leading to a loss of mitochondrial membrane potential, which triggers ferroptosis (Figure 1).[124-126] While HMGB1 has been shown to have increased expression in murine models of DOX induced cardiotoxicity, it is unclear if it is most strongly associated with necrosis, inflammation, ferroptosis, or apoptosis in the cardiotoxic setting.

Apoptosis and necrosis can also be induced by DOX *via* oxidative damage of the mitochondria. DOX increases oxidative stress, which upregulates pro-apoptotic Bnip-3 expression.[120] Increased intracellular levels of Bnip-3 compromise the mitochondrial integrity, causing cardiomyocytes to enter an energy crisis and initiate apoptosis or necrosis (Figure 1).[73] As increasing numbers of cardiomyocytes initiate cell death pathways, it contributes to the randomly distributed cardiomyocyte death characteristic of DOX+TRZ induced cardiomyopathy, ultimately leading to declines in cardiac function.

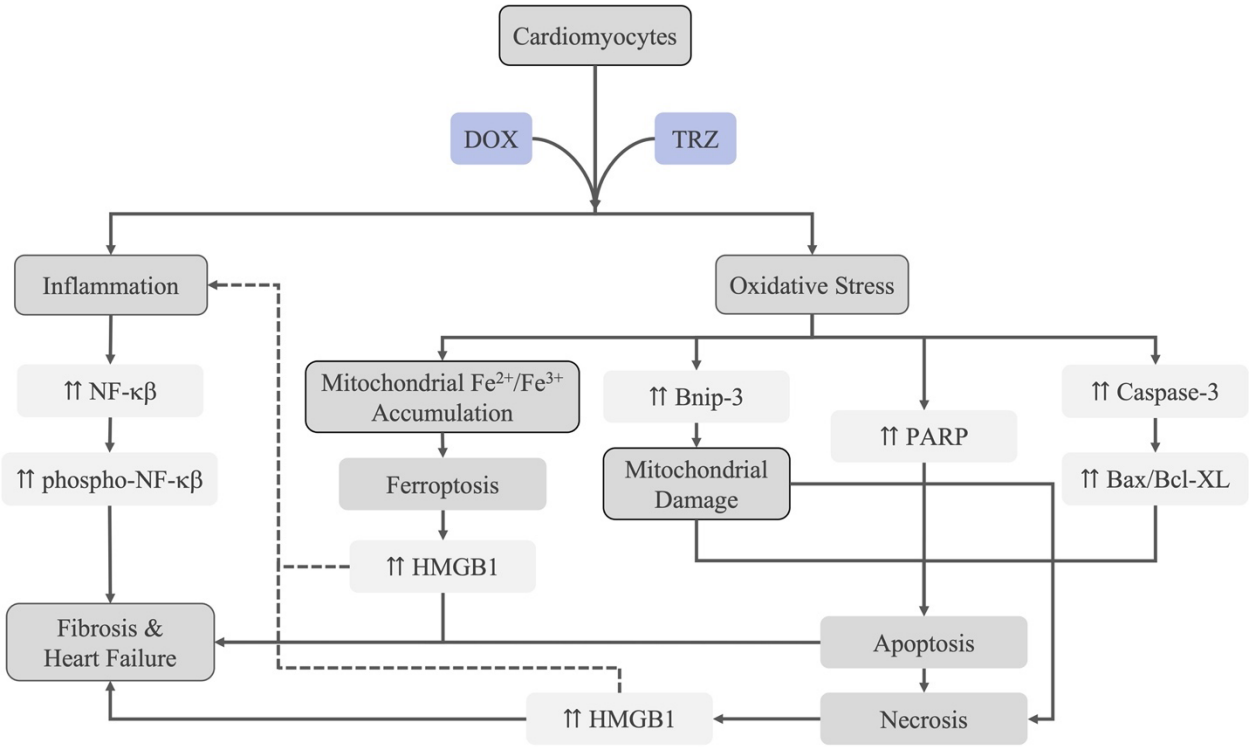

Figure 1. Mechanisms of DOX+TRZ mediated cardiotoxicity. In experimental models of cardiac injury due to DOX+TRZ, pro-inflammatory markers are up-regulated leading to cardiac fibrosis and heart failure. Additionally, DOX+TRZ mediated oxidative stress leads to mitochondrial damage and activation several pro-apoptotic genes leading to increased cell death and cardiotoxicity.

In addition to inducing changes in protein expression, tissue damage can elicit changes in lipid metabolism. The main mediators of polyunsaturated fatty acid (PUFA) effects in the body are oxylipins; products of PUFA oxidation. Oxylipins can be produced from n-3 or n-6 PUFAs *via* the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways.[127] The n-6 PUFA derived oxylipins generally have more inflammatory, vasoconstrictory, and proliferative effects than n-3 PUFA derived oxylipins, although there are exceptions.[127] The CYP pathway involves an array of membrane-bound enzymes that can have epoxygenase or ω-hydroxylase activity. The CYP epoxygenase pathway produces epoxy-docosapentaenoic acid (EpDPE), epoxy-eicosatetraenoic acid (EpETE), or epoxy-eicosatrienoic acid (EpETrE) depending on the substrate.[127] The CYP ω-hydroxylase pathway produces hydroxy-docosahexaenoic acid (HDoHE) from docosahexaenoic acid (n-3), hydroxy-eicosapentanoic acid (HEPE) from eicosapentaenoic acid (n-3), and hydroxy-eicosatetraenoic acid (HETE) from arachidonic acid (n-6). These CYP ω-hydroxylase oxylipins mediate their effects *via* receptors, cross-reactions with other oxylipin receptors, or modulating intracellular transcription factors and ion channels.[127] The arachidonic acid derived CYP oxylipins have renal, vascular, and cardiac functions (Figure 2). For example, 20-HETE has a hypertensive effect in the renal and coronary arteries while 16-, 18-, and 19-HETE, and 20-carboxy-arachidonic acid (20-COOH-AA) can promote vasodilation. 20-HETE can also stimulate inflammatory cytokine production in endothelial cells.[127]

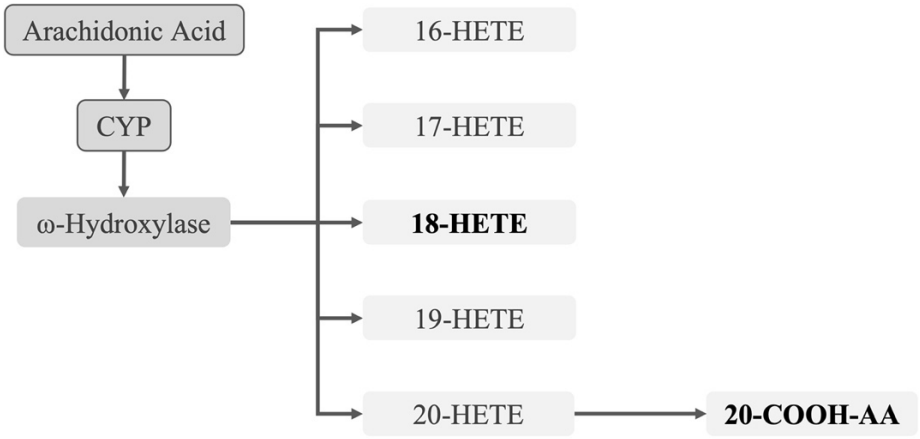

Figure 2. Arachidonic acid-derived oxylipins. The CYP ω-hydroxylase metabolic processing of arachidonic acid produces the oxylipins 16-HETE, 17-HETE, 18-HETE, 19-HETE, 20-HETE, and 20-COOH-AA among other metabolites associated with vascular tone and inflammation.

In the clinical setting, analysis of biomarkers is mostly limited to serum samples. Development of CTRCD has been predicted by early increases in cardiac biomarkers including troponins, natriuretic peptides, and inflammatory proteins.[128-130] Troponins are thin-filament contractile proteins that are present in high concentration within the myocardium.[131] Decreases in the E/A ratio, an echocardiographic marker of diastolic dysfunction, correlates with elevated serum cardiac troponin-T (cTnT) levels in patients receiving anthracycline chemotherapy.[131-136] Elevations in troponin I (TnI) above 0.08ng/mL has also been associated with an increased risk and severity of CTRCD.[131] Natriuretic peptides are produced in the myocardium and are released in response to increased pressure and volume overload.[137] Although the current literature is conflicting, some studies suggest there is a correlation between elevated N-terminal pro-brain type natriuretic peptide (NT-pro-BNP) and/or BNP, and increased risk of developing CTRCD.[46,138-140] Biomarkers of inflammation, including C-reactive protein (CRP) and galectin-3, have also shown some potential in predicting CTRCD, particularly TRZ-mediated cardiotoxicity.[141,142] In a clinical trial

of HER2 positive breast cancer patients receiving TRZ, elevated levels of serum high-sensitivity CRP (≥3mg/L) was predictive of future decreases in LVEF.[142]

1.2.5 Prevention & Treatment of Chemotherapy Mediated Cardiotoxicity

The evolving field of Cardio-Oncology applies various pharmacological therapies in attempt to prevent and/or treat chemotherapy mediated cardiotoxicity. The current pharmaceuticals include antioxidants, β-blockers, statins, and RAS antagonists, with RAS antagonists comprising the most effective option for both the prevention and treatment of chemotherapy mediated cardiotoxicity.[120,128,143-154] RAS antagonists are most effective when used in the pre-treatment setting and are often administered in conjunction with β-blockers to help preserve LVEF.[80]

In the pre-clinical setting, RAS antagonists have been found to attenuate the cardiotoxic changes induced by chemotherapy and decrease mortality.[80,155] Prophylactic administration of the ACEi Perindopril (PER) or the angiotensin receptor blocker (ARB) Valsartan in a chronic *in vivo* murine model of DOX+TRZ mediated mediated cardiotoxicity was shown to attenuate adverse cardiac remodeling and decrease overall mortality (Figure 3).[153,154] The reduced mortality with use of ARBs can be attributed to the cardioprotective effects of decreased blood pressure (BP), increased myocyte fractional shortening, reduced oxidative stress, decreased percentage of apoptotic cells, preserved LVEF, and prevention of adverse cardiovascular remodeling.[80,156] Cardioprotection is more successful with earlier initiation of treatment suggesting that the protective effects may be more pronounced than the treatment effects of RAS antagonists.[80,157,158]

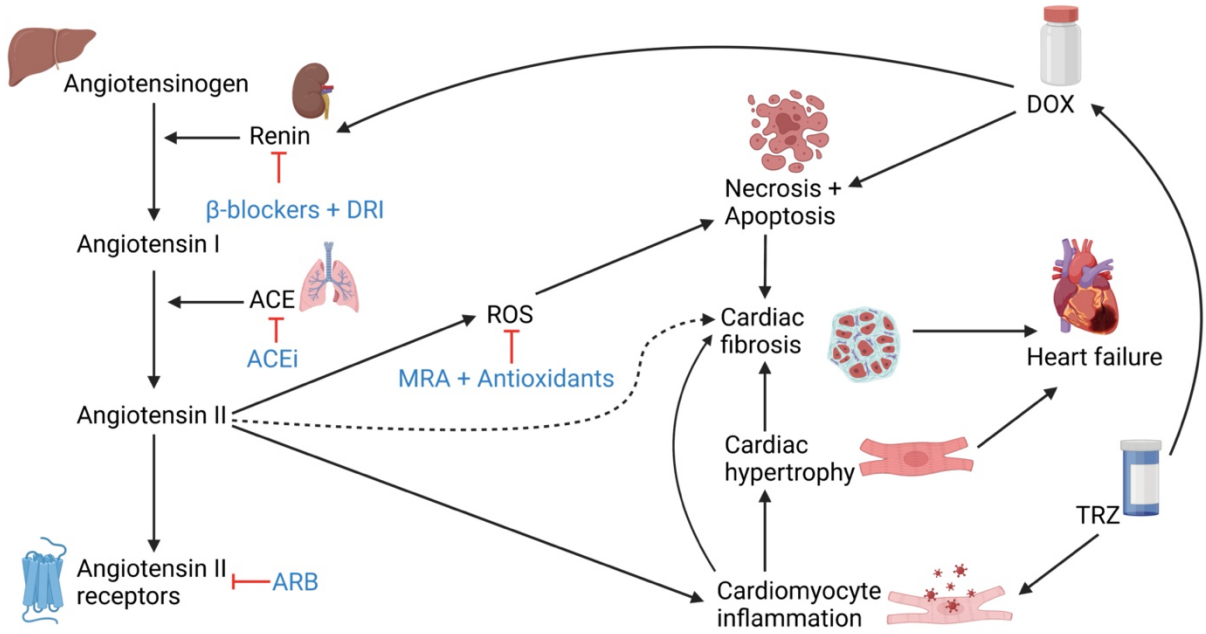

Figure 3. Mechanistic pathways of various pharmacological agents including β-blockers, direct renin inhibitors (DRIs), ACEi, ARBs, and mineralocorticoid receptor antagonists (MRAs) in preventing DOX+TRZ mediated cardiotoxicity (Figure modified from Telles-Langdon *et al.*; see reference 158).

In the clinical setting, retrospective studies and randomized controlled trials have been used to examine the role of RAS inhibition in Cardio-Oncology.[159-163] When considering type I cardiotoxicity, starting ACEi at least 24 hours before treatment with DOX and continuing ACEi for 6 months has been shown to lower incidence of reduced LVEF, heart failure, and death in various cancer settings.[162,163] ARB administration during chemotherapy has also shown beneficial effects in the prevention of early echocardiographic signs of heart failure, oxidative damage, and inflammation. The reduction of oxidative damage is demonstrated by reduced serum levels of ROS, while the decreased inflammation is demonstrated by lower levels of IL-6, IL-1β, TNF-α, and monocyte chemoattractant protein 1 (MCP-1).[80,164-166] Antioxidant effects against anthracycline induced oxidative stress, decreased markers of myocardial injury, and protection of systolic function, diastolic function, and LVEF have also been shown with the use of

mineralocorticoid receptor antagonists (MRAs) in the clinical setting.[80,159] Examining DOX+TRZ mediated cardiotoxicity, both systolic and diastolic dysfunction has been partially prevented through prophylactic treatment with the ACEi PER.[159,160] The PRADA trial found that the ARB Candesartan was also effective for protecting against declines in LV function caused by type I and type II cardiotoxicity.[159] RAS inhibition is therefore an effective treatment for chemotherapy mediated cardiotoxicity since DOX+TRZ are often used in conjunction in the breast cancer setting. Focusing solely on preventing TRZ mediated type II cardiotoxicity, the MANTICORE trial found that the ACEi PER and the β-blocker Bisoprolol were effective in the prevention of a decline in LVEF.[81]

The current clinical standard of care in the breast cancer setting is to avoid prescribing additional pharmaceuticals until they are proven necessary. This means that patients are not prescribed cardioprotective agents until after they develop chemotherapy mediated cardiotoxicity. Following the current Canadian Cardiovascular Society guidelines, once a patient has been diagnosed with chemotherapy mediated cardiotoxicity, marked by a LVEF <53% or a >10% decline in LVEF from baseline, they should receive an ACEi or ARB and a β-blocker.[107] This is conventionally started as a low-dose of PER (2mg/day) and Bisoprolol (2.5mg/day) with adjustments to the prescriptions based on each patient's response to treatment. The goal of these treatments is to reverse the cardiac injury without causing new side effects.[107] However, the current treatment does not maintain its initial effectiveness throughout the duration of treatment and may present unwanted side effects such as hypotension. It is therefore imperative to continue to investigate alternative treatment options for cardiotoxicity.

1.3 Nutraceuticals

Nutraceuticals encompass all foods with a positive health effect beyond basic nutrition. These functional foods contain physiologically active components that have been reported to improve several disease conditions including cancer and cardiovascular disease.[167] The cardioprotective role of nutraceuticals is an evolving area of research in Cardio-Oncology due to the side effects and limited efficacy of the existing pharmaceutical treatments for cardiotoxicity.

1.3.1 Flaxseed

In addition to its basic nutritional function, FLX has been shown to have a positive health effect in several disease conditions including both cancer and cardiovascular disease.[167,168] The physiologically active nutraceutical components found in flax include alpha-linoleic acid (ALA) and secoisolariciresinol diglucoside (SDG). ALA has been shown to have anti-inflammatory effects that may block some cardiotoxic mechanisms of DOX+TRZ.[168] SDG has been shown to have antioxidant properties that may mediate the cellular and mitochondrial mechanisms of DOX+TRZ induced cardiotoxicity (Figure 4).[168] As a nutraceutical, FLX does not present the side effects associated with the pharmaceuticals currently used in the treatment of cardiotoxicity and is therefore worth considering as a possible alternative treatment.

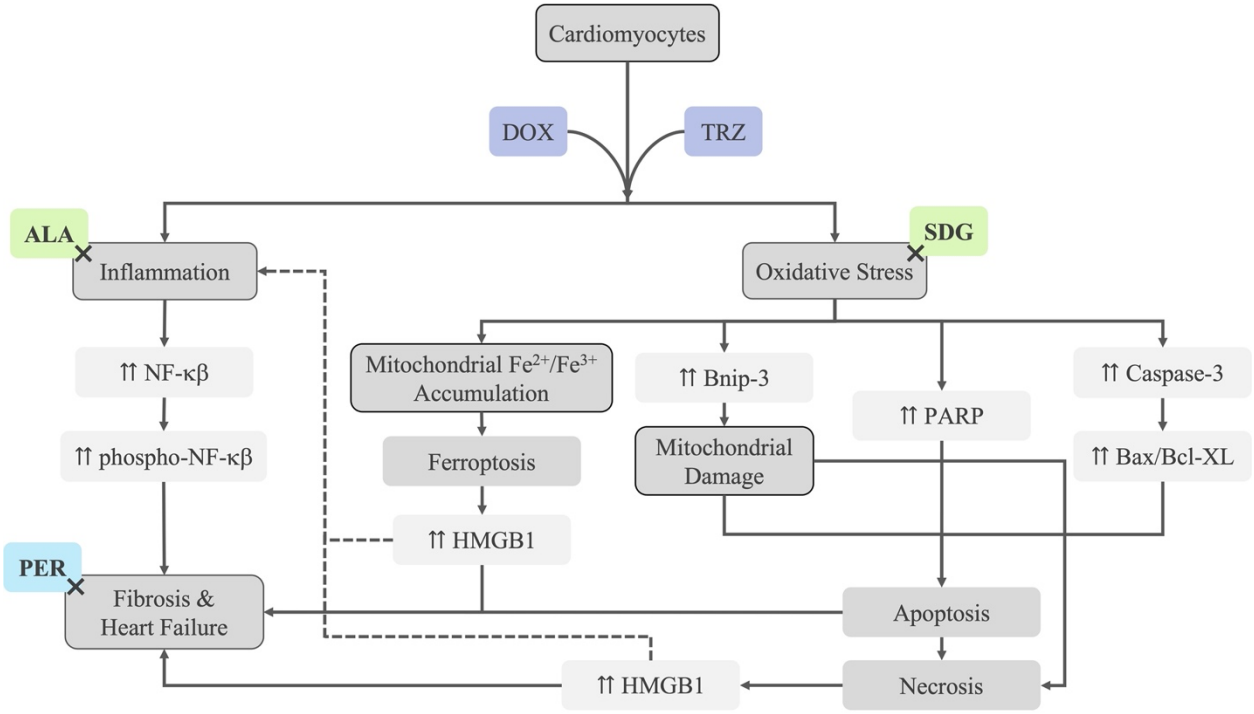

Figure 4. Mechanisms of DOX+TRZ mediated cardiotoxicity and FLX+PER cardioprotection. In experimental models of cardiac injury due to DOX+TRZ, pro-inflammatory markers are up-regulated leading to cardiac fibrosis and heart failure. Additionally, DOX+TRZ mediated oxidative stress leads to mitochondrial damage and activation several pro-apoptotic genes leading to increased cell death and cardiotoxicity. The overall hypothesis is that FLX and its components (ALA and SDG) will treat the cardiotoxic side effects of DOX+TRZ through their anti-inflammatory and antioxidant properties, with similar cardiac outcomes as treatment with ACEi using PER.

1.3.2 Cancer

The effects of dietary FLX consumption on cancer have been investigated in both pre-clinical and clinical trials, showing a protective effect, most notably in the breast cancer setting.[169-171] FLX supplementation can reduce both cancer risk and disease progression.[169,170] In pre-clinical models, SDG and FLX have been shown to support the treatment of cancer by reducing estrogen activity and improving TRZ efficacy, respectively.[172,173] In the high estrogen environment of breast cancer, dietary SDG has a SERM-like anti-estrogen effect that can normalize dysplasia, cell number, and

gene expression in mammary gland tissue.[172] In a murine model of HER2 positive breast cancer, dietary FLX oil enhanced the tumor reducing effects of TRZ.[173]

The efficacy of FLX in the reduction of breast cancer risk has also been established in clinical studies.[174,175] A comparative study showed an association between FLX intake and a reduction in breast cancer risk suggesting a potential role in breast cancer prevention.[174] A randomized, double-blind, placebo-controlled, prospective study found that 30 days of dietary FLX consumption in postmenopausal women with primary breast cancer decreased tumor cell proliferation, increased tumor apoptosis, and increased urinary lignan excretion suggesting that FLX has the potential to reduce tumor growth in patients with breast cancer.[175] Further clinical studies have shown that FLX consumption does not interfere with the cytotoxic abilities of anti-cancer treatments, and may actually potentiate specific anti-cancer agents like TRZ.[173,175-184] Up to 30% of breast cancer patients consume FLX due to the possibility of reducing cancer progression and preventing co-morbidities, without the side-effects associated with pharmaceuticals.[167,174] Therefore, FLX consumption is safe and effective in the breast cancer setting and may have a dual benefit.

1.3.3 Cardiovascular Disease

The effects of dietary FLX consumption on cardiovascular disease have also been investigated in pre-clinical trials, clinical trials, and systematic reviews showing a positive effect in the pathological cascades of atherosclerosis, hypertension, arrythmias, and heart failure. In pre-clinical trials, murine models have demonstrated that FLX, has anti-atherosclerotic effects, demonstrating both plaque prevention and plaque regression, in the presence of high cholesterol or high fat diets.[185-189] A murine model of myocardial infarction (MI) demonstrated that FLX also reduces the

incidence of arrythmias, and decreases infarct size, LV dilation, and myocardial fibrosis, suggesting it may effectively prevent arrythmias and support ventricular remodelling post-MI.[190]

Clinical trials have shown similar results. The FlaxPAD study was a prospective, double-blinded, placebo-controlled, randomized clinical trial that found that FLX significantly decreased both systolic and diastolic BP in patients with peripheral artery disease showing the most potent anti-hypertensive effect ever achieved *via* dietary intervention.[191] Further analysis of the FlaxPAD population found that FLX also significantly decreases systolic and diastolic central aortic blood pressures in patients with hypertension.[192] The anti-hypertensive effects of FLX have been corroborated by several other clinical trials and meta-analyses.[193-199] Another double-blinded, placebo-controlled, randomized clinical trial found that dietary FLX can decrease plasma cholesterol and glucose concentrations in a dose-dependent manner, further supporting the role of FLX in the treatment of vascular diseases.[200] Looking specifically at ALA, reviews have concluded that ALA may be used to reduce heart rate (HR) or as a form of clinical prevention for sudden cardiac death, due to its anti-arrhythmic effects.[201] Overall, there is significant support for the clinical use of dietary FLX in the treatment of cardiovascular disease due to its high content of ALA and SDG contributing to anti-atherosclerotic, anti-arrythmic, and anti-hypertensive effects, all of which are effective components in the treatment of heart failure.[202-204] The existing evidence surrounding the health benefits of FLX in a variety of cardiovascular diseases supports further investigations into possible clinical uses of FLX in other cardiovascular disease contexts.

1.3.4 Flaxseed in the Prevention of Chemotherapy Mediated Cardiotoxicity

While the mechanisms of chemotherapy induced cardiotoxicity are an area of ongoing research, inflammation and oxidative stress are two established mechanistic pathways. The anti-inflammatory and antioxidant properties of ALA and SDG in FLX therefore inhibit these mechanisms and can partially prevent chemotherapy induced cardiotoxicity.[168,205] As recently demonstrated by our lab, prophylactic treatment with FLX, ALA, and SDG were partially cardioprotective in a chronic *in vivo* female mouse model of DOX+TRX mediated cardiotoxicity, and FLX offers equivalent cardioprotection to ACEi.[168,205]

In the first study, mice treated with DOX+TRZ demonstrated a decrease in LVEF from 75±2% at baseline to 37±3% at the study endpoint ($p<0.05$). However, mice treated prophylactically with FLX, ALA, and SDG showed partially attenuated LV function with endpoint LVEF values of 62±2%, 61±3%, and 62±4% respectively ($p<0.05$).[168] Mice treated with DOX+TRZ also showed an approximately 2-fold increase in cardiac tissue biomarkers of inflammation, mitochondrial dysfunction, and apoptosis when compared to healthy controls. These pathological signaling pathway increases were attenuated by prophylactic administration of FLX, ALA, and SDG ($p<0.05$).[168] The study also demonstrated attenuation of DOX+TRZ-induced loss of cellular integrity and myofibril disarray when compared to healthy controls with prophylactic administration of FLX, ALA, and SDG ($p<0.019$, $p<0.033$, and $p<0.002$, respectively) through histological analyses.[168]

Based on the promising results previously described, a second study was conducted to compare the cardioprotective role of FLX to standard pharmacological therapy using the ACEi PER.[205] In

this second study, mice treated with DOX+TRZ demonstrated a decrease in LVEF from 75±2% at baseline to 37±3% at the study endpoint ($p<0.05$). However, mice treated prophylactically with FLX, PER, and FLX+PER showed partially attenuated LV function with endpoint LVEF values of 61±2%, 62±2%, and 64±2% respectively ($p<0.05$).[205] Mice treated with DOX+TRZ also showed a greater than 2-fold increase in cardiac tissue biomarkers of inflammation and oxidative stress, when compared to healthy controls. The increase in these pathological signaling pathways were attenuated by the prophylactic administration of PER, FLX, and FLX+PER ($p<0.05$).[205] The study also demonstrated attenuation of DOX+TRZ-induced loss of sarcomere integrity, myofibril disarray, and vacuolization when compared to healthy controls with prophylactic administration of FLX, PER, and FLX+PER ($p<0.05$) through histological analyses.[205]

Previous evidence has demonstrated the efficacy of prophylactic FLX in the prevention of chemotherapy mediated cardiotoxicity. Considering these findings, in addition to the previously described effect of dietary FLX in the treatment of atherosclerosis, arrythmias, hypertension, and sudden cardiac death, it is reasonable to hypothesize that FLX may be efficacious in the treatment of chemotherapy mediated cardiotoxicity. Little is known on the effects of FLX in the context of Cardio-Oncology, which supports investigation into the role of FLX in the ***treatment*** setting.

Chapter 2: Rationale, Hypothesis, & Objectives

2.1 Rationale

While the combination of DOX+TRZ is a highly effective treatment in the breast cancer setting, the cardiotoxic side effects significantly attenuate its treatment benefits. Up to 25% of patients are at risk of developing DOX+TRZ mediated cardiotoxicity; therefore, treatment strategies for chemotherapy induced cardiotoxicity is of paramount importance in the field of Cardio-Oncology.[106]

The current Canadian Cardiovascular Society guidelines recommend that patients who develop cardiac dysfunction due to chemotherapy be prescribed with low-doses of an ACEi and a β-blocker to mitigate the existing damage and prevent further cardiac deterioration.[107] While these treatments are partially effective, they do not maintain the initial effectiveness throughout the duration of treatment and may present unwanted side effects. We may therefore be able to reduce the side-effects of the treatment of cardiotoxicity by complementing pharmaceuticals with nutraceuticals.

There is growing scientific evidence to support the use of dietary FLX as treatment for a variety of health conditions.[202] In breast cancer patients, FLX has been shown to both prevent development and reduce tumor progression.[169] Recently, our lab has demonstrated the cardioprotective effects of FLX components and that FLX is equivalent to PER in the prevention of chemotherapy induced cardiotoxicity in a chronic *in vivo* murine model.[168,205] Despite the efficacy of a cardioprotective effect, the prevention timeline does not align with the clinical presentation. Typically, breast cancer patients present to a cardiologist for treatment following the end of their cancer treatment after CTRCD is already well established. Therefore, a treatment model is more applicable to the

clinical setting. However, little is known about whether the administration of FLX is equivalent and/or synergistic with PER in the treatment of chemotherapy induced cardiotoxicity.

2.2 Hypothesis

The effects of dietary FLX will be comparable to and/or synergistic with conventional PER in the treatment of DOX+TRZ mediated cardiotoxicity in an *in vivo* murine model of DOX+TRZ induced cardiomyopathy.

2.3 Objective

The specific aim is to evaluate whether FLX is comparable and/or incremental to standard pharmacological therapy using the ACEi PER in the treatment of DOX+TRZ mediated cardiotoxicity in a chronic *in vivo* murine model.

Chapter 3: Methods

3.1 Animal Model

The guidelines of the Canadian Council on Animal Care were strictly followed for all animal procedures, including drug administrations and longitudinal echocardiographic studies, as approved by the Animal Protocol Review Committee at the University of Manitoba [REB: 20-004/1 (AC11548)].

A total of 110 wild-type C57Bl/6 female mice (12-15 weeks old; Jackson Laboratories, Bar Harbor, ME, US) were used for the purposes of this study. They had *ad libitum* access to water and the study diets, maintained on a 12-hour day/night cycle while being housed in cages of 2 to 3 mice in the animal holding facility. At weeks 1, 2, and 3, the mice received weekly intraperitoneal (i.p.) injections with either saline or DOX+TRZ (8mg/kg and 3mg/kg, respectively)[206] in pre-specified groups to create a chronic *in vivo* murine model of chemotherapy induced cardiotoxicity. Mice had *ad libitum* access to regular chow or 10% FLX (~0.5g/day) supplemented diet on a daily basis starting on week 4 for an additional 3 weeks (total of 3 weeks). As RAS antagonism using an ACEi is used in the treatment of chemotherapy mediated cardiotoxicity, mice were also administered PER (3mg/kg/day) *via* oral gavage from week 4 to the study endpoint in the pre-specified groups (Figure 5). All mice were subject to weekly echocardiograms at baseline and the 6 experimental weeks (Figure 6) as well as hemodynamic measurements at baseline, midpoint, and endpoint, and weight analysis throughout the study.

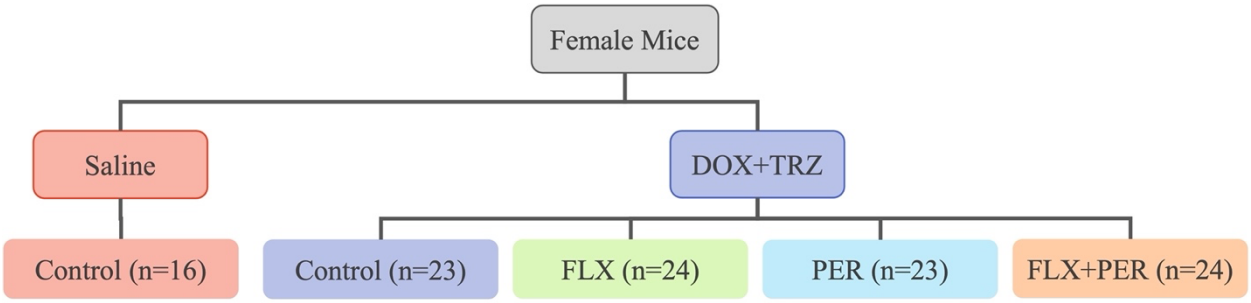

Figure 5. Experimental randomization. A total of 110 WT C57Bl/6 female mice (12-15 weeks old; Jackson Laboratories, ME, US) were randomized into control or the DOX+TRZ anti-cancer treatment group receiving: 0.9% saline (n=16) or 8mg/kg DOX + 3mg/kg TRZ (n=94) administered at the start of week 1, 2, and 3 *via* i.p. injection in order to produce a chronic *in vivo* murine model of chemotherapy induced cardiotoxicity. Mice were further randomized to receive *ad libitum* access to their respective diets and PER or water *via* oral gavage for weeks 4 through 6.

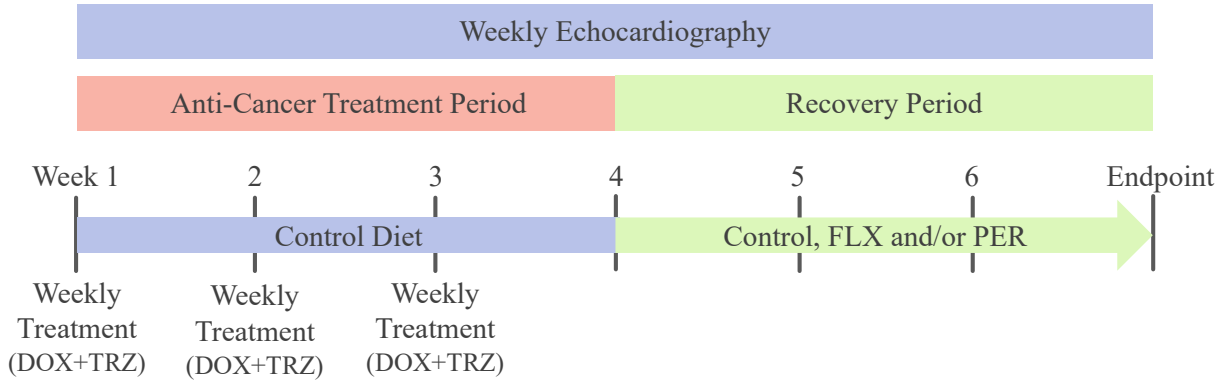

Figure 6. Experimental timeline. Mice were randomized into control or the DOX+TRZ anti-cancer treatment group receiving: 0.9% saline (n=16) or 8mg/kg DOX + 3mg/kg TRZ (n=94) administered at the start of week 1, 2, and 3 *via* i.p. injection in order to produce a chronic *in vivo* murine model of chemotherapy induced cardiotoxicity. Mice were further randomized to receive *ad libitum* access to their respective diets and PER or water *via* oral gavage for weeks 4 through 6. Cardiac function was assessed weekly using non-invasive TTE.

3.2 Murine Echocardiography

Serial non-invasive TTEs were performed weekly on awake mice at baseline and for the 6 experimental weeks to assess cardiac function and cardiovascular remodeling.[120,165,170,207-212] A 13-MHz linear array ultrasound probe (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US) was used to capture the images that were later analyzed using the EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US). Images acquired in the parasternal long axis (PLAX) view were analyzed to calculate LVEF based on the endocardial borders and application of the Teicholtz formula (Figure 7). Images acquired in the parasternal short axis (PSAX) M-mode view were analyzed to calculate HR, LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), posterior wall thickness (PWT), interventricular septal wall thickness (IVS), and LVEF using the Simpson formula (Figure 8). Images acquired in the PSAX view with tissue doppler imaging (TDI) were analyzed to calculate cardiomyocyte contraction velocity.[151,213]

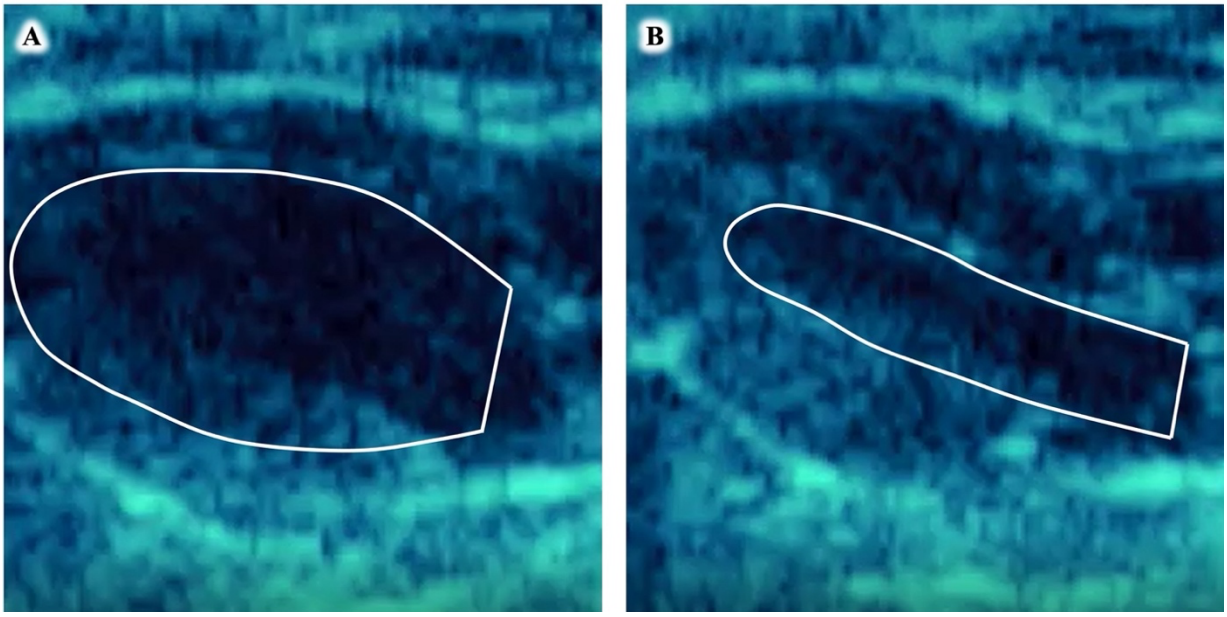

Figure 7. Parasternal long axis view on 2D transthoracic murine echocardiography. LV endocardial border delineation on EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US) for calculation of LVEF using the Teicholtz formula. **Panel A:** Endocardial border tracing at end diastole. **Panel B:** Endocardial border tracing at end systole.

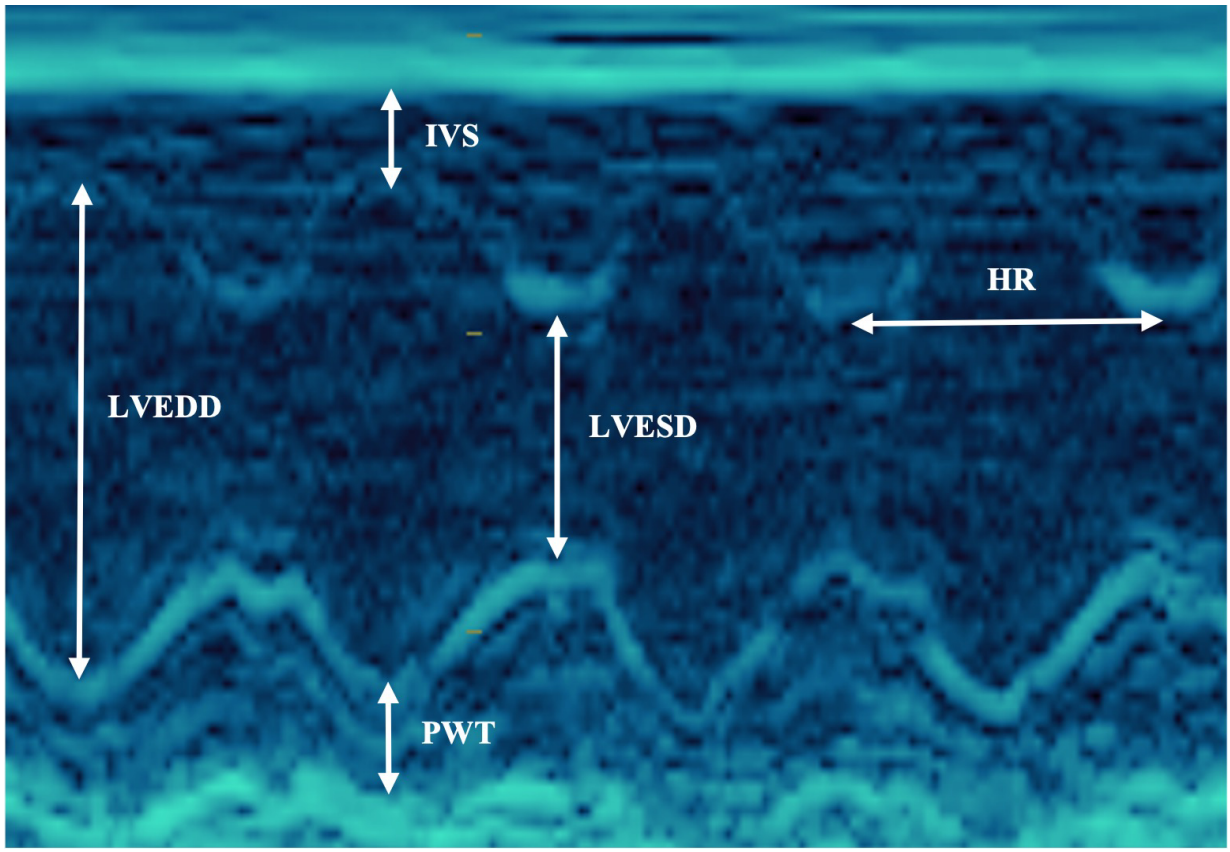

Figure 8. M-mode parasternal short axis view on 2D transthoracic murine echocardiography. LV cavity dimensions and HR as measured using M-mode on EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US).

HR, Heart rate; IVS, Interventricular septum; LVEDD, Left ventricular end-diastolic diameter; LVESD, Left ventricular end-systolic diameter; PWT, Posterior wall thickness.

3.3 Histology

LV tissue was processed and used for histological analysis according to procedures established in the lab.[208] Tissue for light microscopy was sectioned and preserved in a 10% neutral buffered formalin solution for a maximum of 5 days prior to paraffin embedding and imaging. Serial 5µm thick sections were cut from each heart for dewaxing, rehydration, and staining with Masson's Trichrome solution. This stain detects fibrosis by staining the normal myofiber red and the collagen blue. Digital images were then taken with identical exposure settings for all sections.

Tissue for electron microscopy was sectioned, cut into 0.5mm^2 pieces, fixed in a 1:1 ratio of 0.2M PO_4 buffer and 5% glutaraldehyde for 3 hours, then rinsed overnight at 4°C in a 5% sucrose in PO_4 buffer, followed by post-fixation with 1% osmium tetroxide in 0.1M phosphate buffer for 2 hours at room temperature. Tissues were then dehydrated in increasing ethanol concentrations and embedded in Epon 812.[214] Finally, tissue sections were stained with uranyl acetate and lead citrate. To avoid observer bias, grids were coded without prior knowledge of their source. Digital images were then taken with the Philips CM12 electron microscope in order to determine the degree of cellular integrity.

For histological analysis, Mann-Whitney and Kruskal-Wallis tests were applied for non-parametric comparison of scores between each group. The scores ranged from 0 to 5, with 0 representing no tissue injury and 5 representing severe damage.

3.4 Hemodynamics

BP was measured at baseline, week 3, and week 6 in restrained, non-sedated mice using the non-invasive tail cuff method (CODA system, Kent Scientific, Torrington, Torrington CT) on a platform heated to 30°C. The mice underwent 3 consecutive days of BP training to become accustomed to the tail cuff system prior to measurement. Measurements included systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), and mean arterial pressure (MAP). 20 consecutive readings were recorded at 1-minute intervals, and the mean scores of a minimum of 9 true readings were used for data analysis.

SBP and DBP were taken as direct measurements from the tail cuff system. PP was calculated from the systolic and diastolic readings using Equation 1. MAP was calculated from the calculated PP and diastolic readings using Equation 2.

Equation 1. Pulse pressure.

$$\text{Pulse pressure} = \text{Systolic blood pressure} - \text{Diastolic blood pressure}$$

Equation 2. Mean arterial pressure.

$$\text{Mean arterial pressure} = (\text{Pulse pressure} / 3) + \text{Diastolic pressure}$$

3.5 Western Blot Analyses

Western blot analysis was performed to quantify markers of oxidative stress and apoptosis (PARP, Bax/Bcl-XL, and Caspase-3), inflammation (NF-κβ and phospho-NF-κβ), mitochondrial dysfunction (Bnip-3), and necrosis (HMGB1) using a specific antibody for each protein.

LV tissue samples were flash frozen in liquid nitrogen then crushed and homogenized in a radioimmunoprecipitation assay (RIPA) lysis buffer containing phosphatase inhibitor (Product #: A32957, Thermo Scientific) and protease inhibitor (Product #: A32965, Thermo Scientific) to isolate total cellular protein while preventing protein degradation. Following a 30-minute incubation period on ice, the samples were centrifuged to remove cellular debris and the supernatants were collected. A Bradford protein assay was then performed to determine protein concentrations using Coomassie Blue Protein Assay Reagent (Product #: 1856209, Thermo Scientific) and comparison to bovine serum albumin (BSA) standards (Product #: 23209, Thermo

Scientific). The Bradford protein assay results were used to make samples containing 30µg of protein, lamelli sample buffer (product number 1610747) dye diluted with 2-mercaptoethanol (product number M6250-100ML, Aldrich Chemistry), and autoclaved water for western blot analysis.

Samples were run in a 5% stacking and an 8%, 10%, or 12% resolving sodium dodecyl sulfate (SDS) polyacrylamide gel. Electrophoresis was employed for 90 minutes at 55mA and 15°C to separate the proteins in each sample. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane with a 0.2µm pore size (Product #: 88520, Thermo Scientific) for 60 minutes at 100V and 7°C. Once the proteins were transferred, the membranes were blocked using 5% skim milk powder (SMP) or BSA in 1x Tris Buffered Saline with 0.1% Tween 20 (product number 0777-1L, VWR) for 60 minutes at 20°C, probed using rabbit-source target-specific primary antibodies for 16 hours at 4°C (Table 3) followed by 1/5000 horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (product number 1706515, BioRad) for 60 minutes at 20°C to enable protein band visualization.

Antibody	Product Number	Dilution	Gel Percentage	Probing Solution
Poly-ADP-ribose polymerase (PARP)	9542S, New England Biolab	1/500	8%	2.5% SMP
Bcl-2 associated X protein (Bax)	2772S, New England Biolab	1/500	12%	2.5% SMP
B-cell lymphoma extra-large (Bcl-XL)	2762S, New England Biolab	1/500	12%	2.5% SMP
Caspase-3	9662S, New England Biolab	1/500	12%	2.5% SMP
Nuclear factor kappa beta (NF-κβ)	8242S, New England Biolab	1/1000	10%	5% BSA
phospho-NF-κβ	3031S, New England Biolab	1/1000	10%	5% BSA
Bcl-2 interacting protein 3 (Bnip-3)	3769S, New England Biolab	1/1000	12%	2.5% SMP
High mobility group box 1 (HMGB1)	3935S, New England Biolab	1/1000	12%	2.5% SMP

Table 3. Western gel percentage and probing conditions for each target protein. Target-specific antibodies, antibody product numbers, antibody dilutions, SDS polyacrylamide electrophoresis gel concentrations, and probing solution concentrations.

In addition to standardization of the protein samples to 30μg using the Bradford protein assay, the results were standardized using a loading control and a matched sample between gels. As the loading control, each membrane was probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibodies for 120 minutes at 20°C followed by 1/5000 horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies for 45 minutes at 20°C to enable visualization. Protein band intensity was normalized to the GAPDH loading control prior to analysis. Since there were more samples being analyzed than could be loaded into a single gel, an additional matched sample was loaded into all gels so that different gels with the same target proteins could be standardized to each other prior to comparison.

Following each incubation in secondary antibodies, protein detection was accomplished using enhanced chemiluminescence (ECL) western blotting substrate (Product #: 32106, Thermo Scientific). The chemiluminescence was imaged using a BioRad ChemiDoc Imaging System

(Image Lab Touch Software, Version 2.4.0.03). Protein band intensity was then quantified by Densitometric analysis using Image Lab 5.2.1 software (BioRad).

3.6 Oxylipins

At the study endpoint, plasma samples were collected for oxylipin analysis. Blood samples were collected into EDTA coated blood collection tubes to prevent coagulation. After resting on ice for 15 minutes, samples were centrifuged for 5 minutes at 4°C and 2000g to remove the blood cells, then the plasma was allocated into 1.5mL sterile plasma tubes and stored at -80°C until processing.

To prepare the plasma samples for oxylipin analysis using high performance liquid chromatography (HPLC), 50µL of plasma, 1mL of water at pH 3.0, 20µL of 10x Schebb's antioxidant, 100µL of internal standard, and 170µL of methanol were aliquoted into 2mL Eppendorf tubes, then vortexed. Once mixed, the samples were tested using pH indicator strips then adjusted to pH 3.0 if necessary, using 1N hydrogen chloride (HCl) and 1N sodium hydroxide (NaOH). Debris was removed from the prepared samples in a centrifuge at 14000 RCF at 4°C for 10 minutes. Oxylipins were then extracted from the samples using Strata-X SPE (Phenomenex, 33µ, 60 mg/3 mL) columns. Before loading the samples, the columns were conditioned by aliquoting 3mL of methanol through each column then equilibrated by aliquoting 3mL of pH 3.0 water through each column using a 10mL syringe. Samples were then loaded from the 2mL Eppendorf tubes into the 3mL columns. To collect any remaining sample, 1mL of 10% methanol in pH 3.0 water was added to the 2mL Eppendorf tubes, vortexed, centrifuged at 14000 RCF at 4°C for 5 minutes, then added to the columns and pushed through as previously described until the columns were dry. The columns were then further dried by quickly pushing through 1mL of

Hexane. Once dried, the columns were eluted into 1.5mL microtubes using 1mL of pressurized methanol that was allowed to enter the sorbent. After soaking for 1 minute, the methanol was pushed through to collect the samples. The samples were then dried under N_2 set to gently blow on the surface of the samples in an evaporator at 37°C for 60 to 90 minutes. Once dry, 100µL of cold solvent A (water-acetonitrile-formic acid, 70:30:0.02 v/v/v) was immediately added to the dry samples, then vortexed and centrifuged at 14000 RCF at 4°C for 10 minutes. The supernatant was then transferred into labelled GC/LC vials already containing a 200µL polypropylene conical insert then run on the liquid chromatography mass spectrometer. Samples were analyzed by high-performance liquid chromatography-electrospray ionization-mass spectroscopy.[215]

3.7 Statistical Analysis

The statistical software packages SPSS 15.0, SPSS version 24, and Graphpad Prism 5 were utilized to perform the statistical analyses. All data are expressed as mean ± standard deviation (SD) unless otherwise noted. Results with $p<0.05$ were considered significant. Echocardiographic analyses were performed by analysis of variance (ANOVA) with Dunnet's post-hoc analysis. Histological analysis was performed using Mann-Whitney and Kruskal-Wallis tests for non-parametric comparison of scores between each group. The scores ranged from 0 to 5, with 0 representing no tissue injury and 5 representing severe damage. Hemodynamic analyses were performed by ANOVA with Dunnet's post-hoc analysis. Western analysis data is expressed as mean ± standard error of the mean (SEM). For post hoc analysis, repeated measures of one-way ANOVA were used to evaluate for significance between independent factors. Statistical significance for the oxidized phospholipid and oxylipin analyses were calculated by one-way ANOVA followed by a Tukey post-hoc test.

Chapter 4: Results

4.1 Murine Echocardiography

For all study groups, HR and LV wall thickness dimensions (IVS and PWT) were similar at baseline and at week 6. Mice treated with DOX+TRZ demonstrated adverse LV structural changes with an increase in LVEDD from 2.9±0.1mm at baseline to 4.2±0.2mm at week 6 ($p<0.05$). Treatment with either FLX, PER, or FLX+PER improved LV structure with LVEDD values of 3.5±0.1mm, 3.4±0.2mm, and 3.4±0.1mm, respectively, at study end point (Table 4). Treatment with the combination of FLX+PER, however, was not synergistic in reversing LV structural changes in mice treated with DOX+TRZ (Figure 9).

Mice treated with DOX+TRZ demonstrated severe LV systolic dysfunction with a decline in LVEF from 74±4% at baseline to 39±5% at week 6 ($p<0.05$). Treatment with either FLX, PER, or FLX+PER improved LV systolic function with LVEF values of 63±4%, 64±3%, and 65±4%, respectively, at study end point (Table 4). Treatment with the combination of FLX+PER, however, was not synergistic in reversing LV systolic dysfunction in mice treated with DOX+TRZ (Figure 10).

Parameter	Group	Baseline	Week 6	p-value
HR (bpm)	Control (n=15)	690±5	692±4	0.88
	DOX+TRZ (n=15)	699±4	701±6	0.91
	FLX+DOX+TRZ (n=15)	686±7	689±5	0.84
	PER+DOX+TRZ (n=15)	690±5	685±7	0.83
	FLX+PER+DOX+TRZ (n=15)	688±6	691±4	0.85
IVS (mm)	Control (n=15)	0.81±0.01	0.81±0.02	0.93
	DOX+TRZ (n=15)	0.82±0.02	0.82±0.03	0.92
	FLX+DOX+TRZ (n=15)	0.81±0.01	0.82±0.01	0.92
	PER+DOX+TRZ (n=15)	0.82±0.01	0.82±0.02	0.97
	FLX+PER+DOX+TRZ (n=15)	0.81±0.01	0.82±0.01	0.89
PWT (mm)	Control (n=15)	0.82±0.01	0.82±0.02	0.91
	DOX+TRZ (n=15)	0.81±0.02	0.81±0.03	0.90
	FLX+DOX+TRZ (n=15)	0.81±0.01	0.80±0.01	0.91
	PER+DOX+TRZ (n=15)	0.80±0.01	0.81±0.02	0.95
	FLX+PER+DOX+TRZ (n=15)	0.82±0.01	0.81±0.01	0.92
LVEDD (mm)	Control (n=15)	2.8±0.1	2.9±0.1	0.89
	DOX+TRZ (n=15)	2.9±0.1	4.2±0.2*	<0.05
	FLX+DOX+TRZ (n=15)	2.8±0.2	3.5±0.1*#	<0.05
	PER+DOX+TRZ (n=15)	2.9±0.1	3.4±0.2*#	<0.05
	FLX+PER+DOX+TRZ (n=15)	2.8±0.2	3.4±0.1*#	<0.05
LVEF (%)	Control (n=15)	73±3	74±5	0.92
	DOX+TRZ (n=15)	74±4	39±5*	<0.05
	FLX+DOX+TRZ (n=15)	74±4	63±4*#	<0.05
	PER+DOX+TRZ (n=15)	75±3	64±3*#	<0.05
	FLX+PER+DOX+TRZ (n=15)	73±4	65±4*#	<0.05

Table 4. Echocardiographic parameters of C57Bl/6 mice receiving Saline or DOX+TRZ followed by daily treatment with FLX, PER, or FLX+PER. Baseline and week 6 measures with p-values.

DOX, Doxorubicin; FLX, Flaxseed; HR, Heart rate; IVS, Interventricular septum; LVEDD, Left ventricular end-diastolic diameter; LVEF, Left ventricular ejection fraction; PER, Perindopril; PWT, Posterior wall thickness; TRZ, Trastuzumab.

The values are presented as mean ± SD. *$p<0.05$ DOX+TRZ vs. Control. *#$p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. DOX+TRZ & Control.

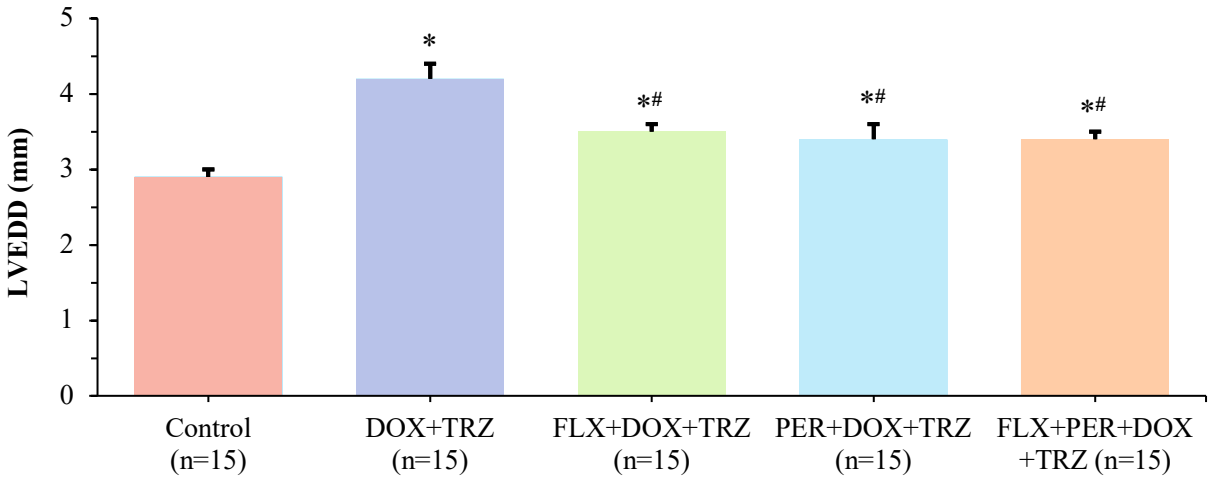

Figure 9. Echocardiographic changes in LVEDD of mice administered with FLX, PER, or FLX+PER after treatment with DOX+TRZ. *p<0.05 DOX+TRZ vs. Control. *#p<0.05 FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. DOX+TRZ and Control.

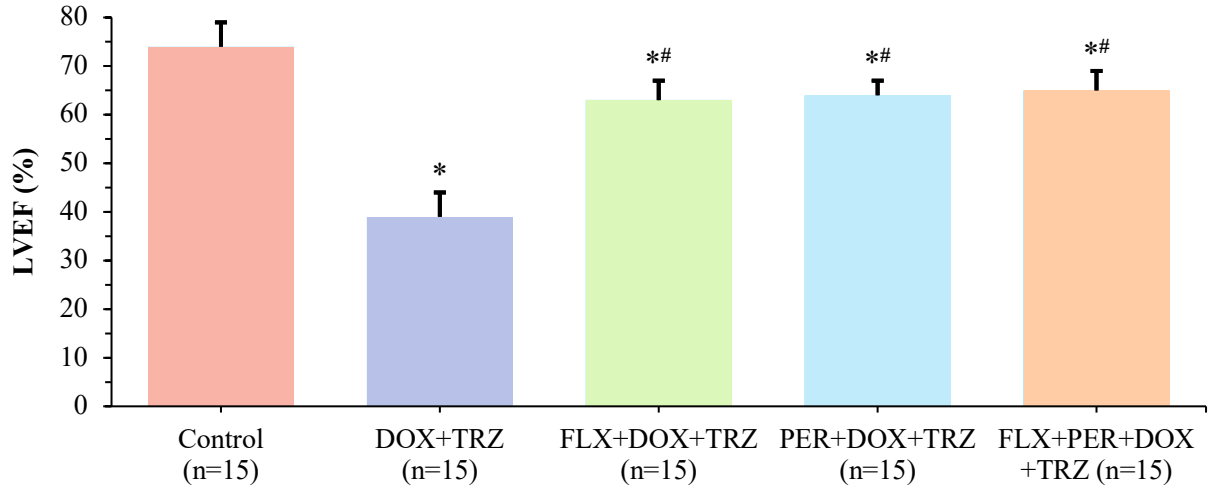

Figure 10. Echocardiographic changes in LVEF of mice administered with FLX, PER, or FLX+PER after treatment with DOX+TRZ. *p<0.05 DOX+TRZ vs. Control. *#p<0.05 FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. DOX+TRZ and Control.

4.2 Histology

Histological analysis of tissue samples was achieved through light microscopy using Masson's trichrome staining and through electron microscopy. Light microscopy showed normal cardiomyocyte integrity in the control group (Figure 11A). In mice treated with DOX+TRZ, there was marked cellular damage and myofibril disarray (Figure 11B). Treatment with FLX, PER, or FLX+PER reduced these histopathological changes (Figures 11C-11E) as compared to treatment with DOX+TRZ ($p<0.05$).

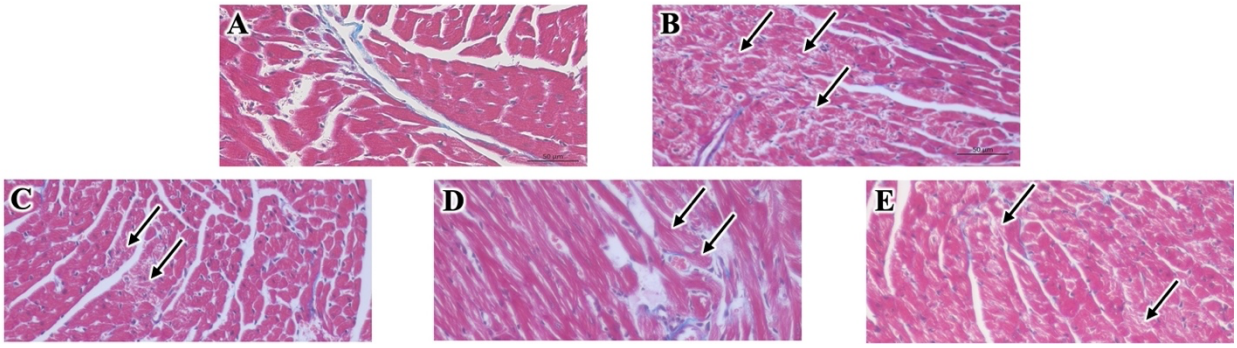

Figure 11. Masson's trichrome stained light microscopy slides representative of the cardiomyocyte morphology changes for each treatment group. Control (A), DOX+TRZ (B), FLX+DOX+TRZ (C), PER+DOX+TRZ (D), and FLX+PER+DOX+TRZ (E).

Electron microscopy also demonstrated normal cardiomyocyte integrity in the control group (Figure 12A). In mice treated with DOX+TRZ, myofibril degradation, vacuolization, and loss of sarcomere integrity was significantly higher as compared to control ($p<0.001$) (Figure 12B). Treatment with FLX, PER, or FLX+PER partially reduced these adverse histological changes (Figures 12C-12E) as compared to the DOX+TRZ group.

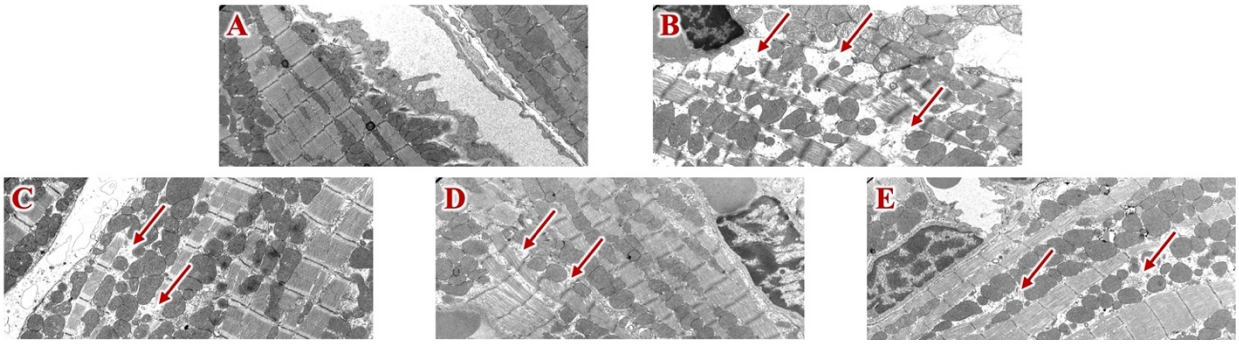

Figure 12. Electron microscopy slides representative of the cardiomyocyte morphology changes for each treatment group. Control (A), DOX+TRZ (B), FLX+DOX+TRZ (C), PER+DOX+TRZ (D), and FLX+PER+DOX+TRZ (E).

4.3 Hemodynamics

There were no statistically significant differences in MAP at week 6 compared to baseline in all study animals (p=NS). Additionally, administration of FLX, PER, or FLX+PER did not significantly alter MAP at week 6 (data not shown).

4.4 Western Blot Analyses

In mice treated with DOX+TRZ, there was a 2.3-fold increase in HMGB1 expression as compared to healthy control mice ($p<0.05$) (Figure 13). Elevations in this necrosis biomarker were significantly downregulated in mice treated with FLX, PER, or FLX+PER ($p<0.05$).

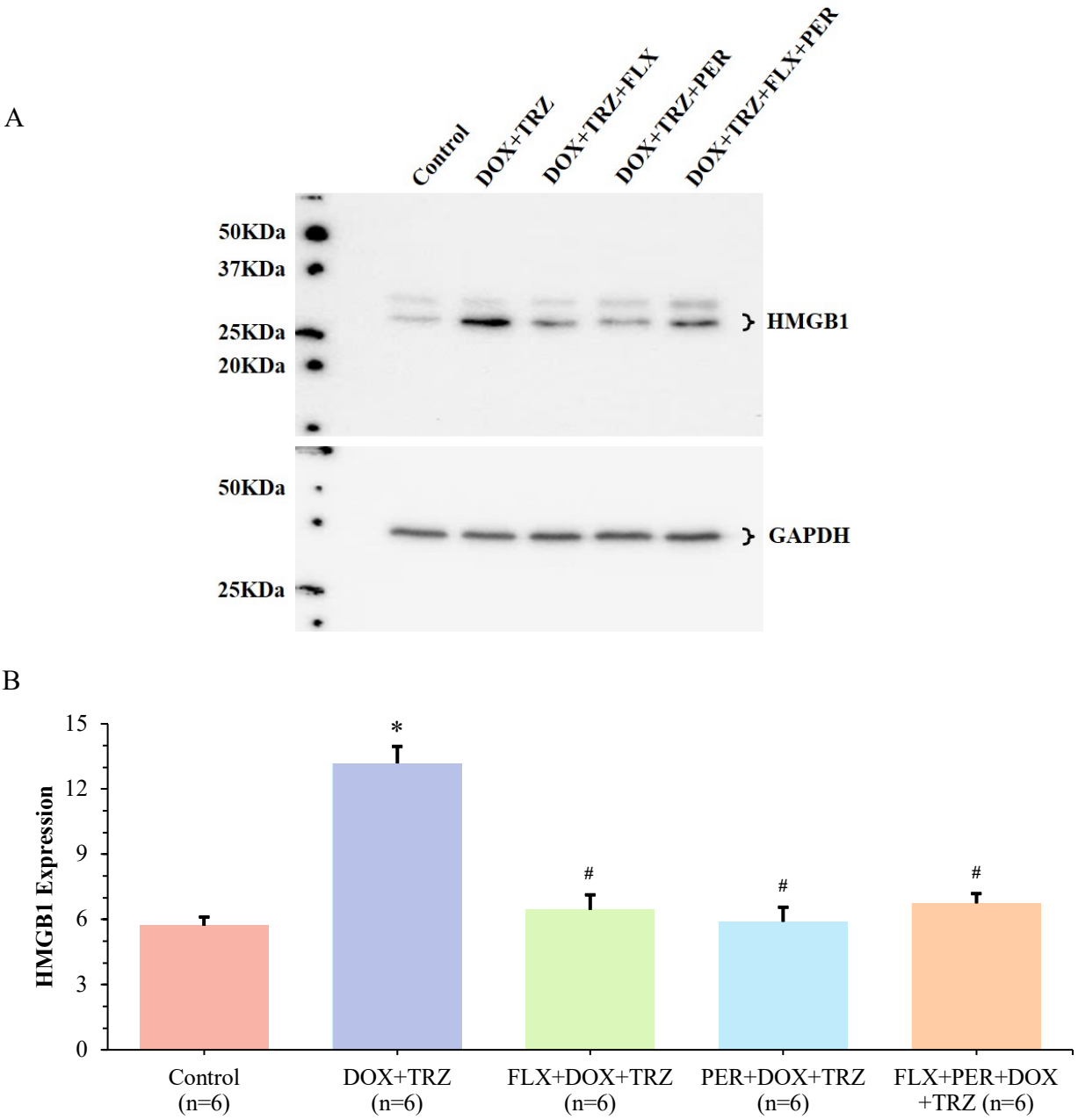

Figure 13. Western blot HMGB1 expression. *$p<0.05$ DOX+TRZ vs. Control. #$p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. DOX+TRZ.

In mice treated with DOX+TRZ, there was a 2.4-fold increase in Bnip-3 expression as compared to healthy control mice ($p<0.05$) (Figure 14). Elevations in this mitochondrial biomarker were significantly downregulated in mice treated with FLX, PER, or FLX+PER ($p<0.05$).

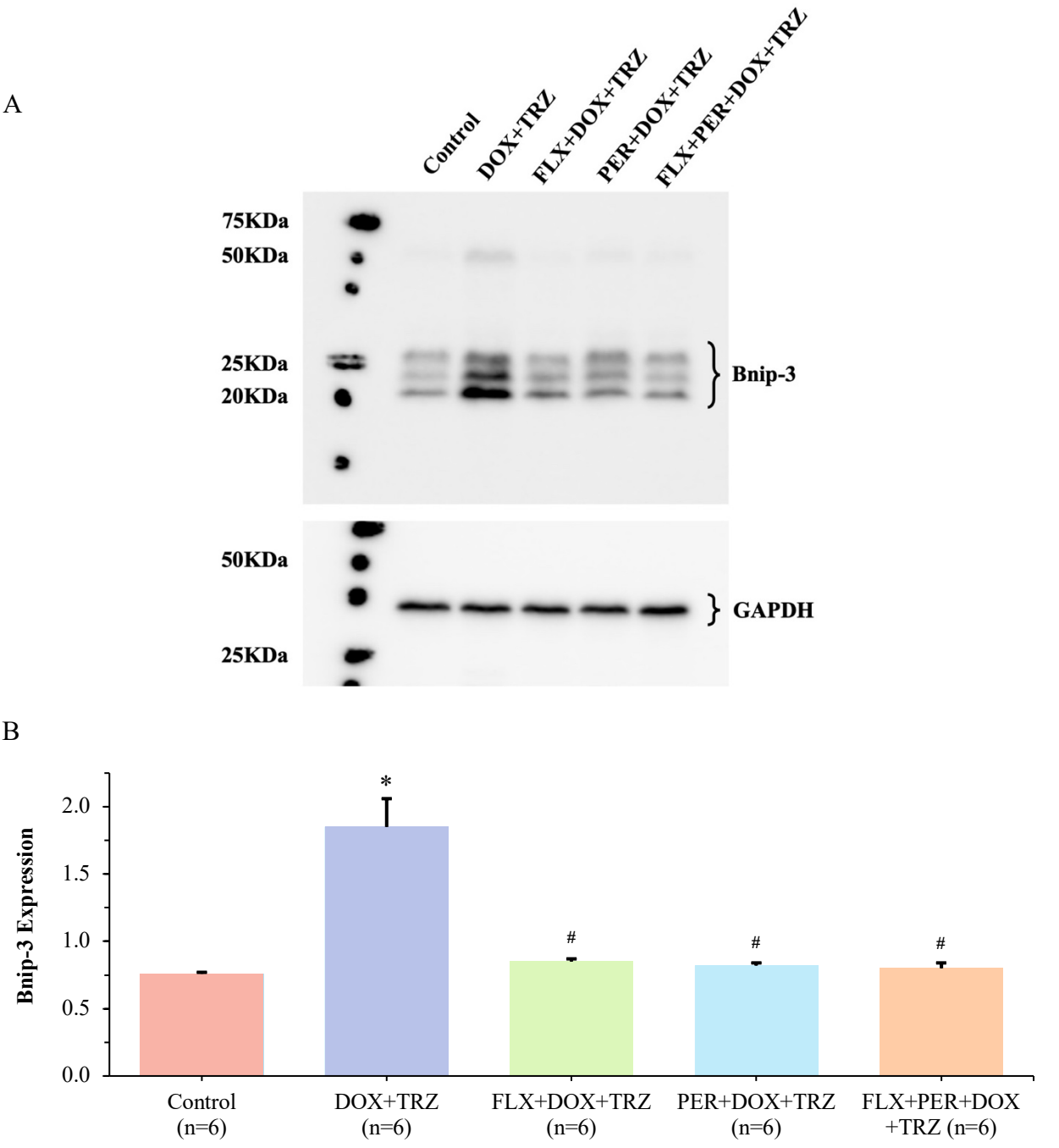

Figure 14. Western blot Bnip-3 expression. *$p<0.05$ DOX+TRZ vs. Control. #$p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. DOX+TRZ.

4.5 Oxylipins

In mice treated with DOX+TRZ, there was a 3.3-fold increase in 18-HETE net calculated concentration as compared to healthy control mice ($p<0.05$) (Figure 15). Elevations in this oxylipin were significantly downregulated in mice treated with FLX, PER, or FLX+PER ($p<0.05$).

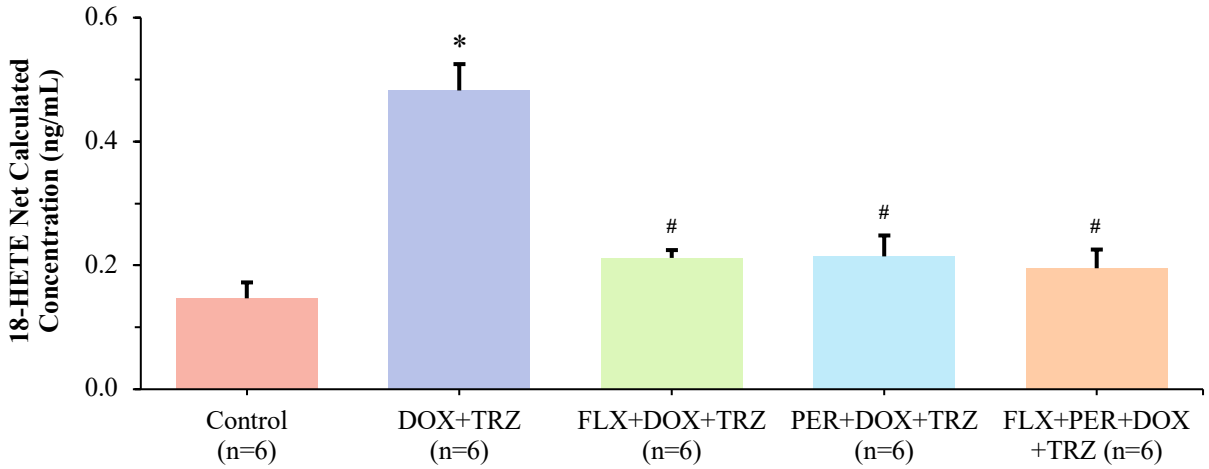

Figure 15. Oxylipin 18-HETE net calculated concentration. *$p<0.05$ DOX+TRZ vs. Control. #$p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. DOX+TRZ.

In mice treated with DOX+TRZ, there was a 7.3-fold increase in 20-COOH-AA net calculated concentration as compared to healthy control mice ($p<0.05$) (Figure 16). Elevations in this oxylipin were significantly downregulated in mice treated with FLX, PER, or FLX+PER ($p<0.05$).

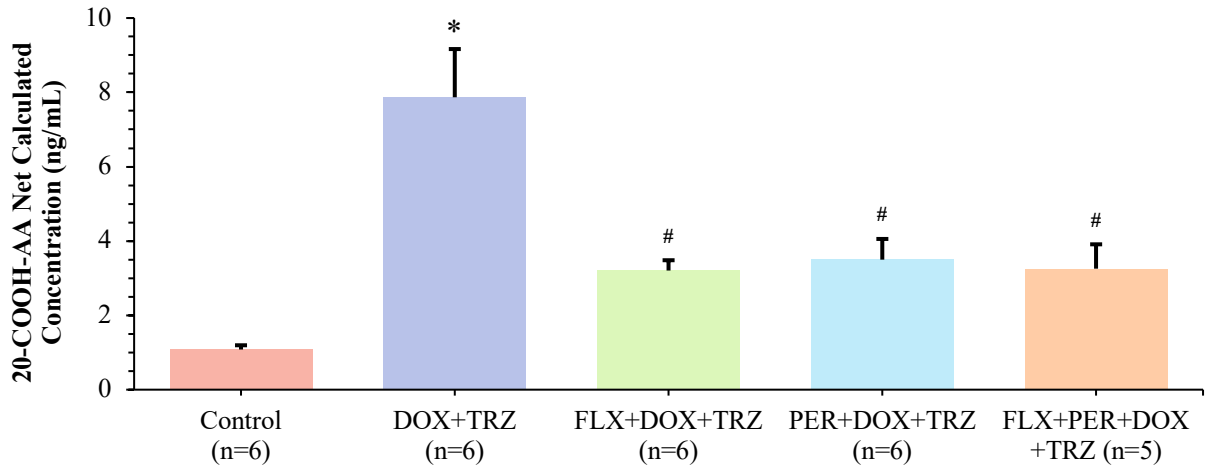

Figure 16. Oxylipin 20-COOH-AA net calculated concentration. *$p<0.05$ DOX+TRZ vs. Control. #$p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. DOX+TRZ.

Chapter 5: Discussion

The evolving field of Cardio-Oncology applies various therapies, both pharmaceuticals and nutraceuticals, in an attempt to treat chemotherapy mediated cardiotoxicity. In our current chronic *in vivo* murine model of DOX+TRZ induced cardiotoxicity, FLX was equivalent to PER in the treatment of adverse LV remodeling, but the combination of FLX+PER was not synergistic. The echocardiographic, histologic, proteomic, and lipidomic parameters offer insight into the degree of attenuation and the mechanisms of the treatment effect. Our study demonstrated that treating established DOX+TRZ induced cardiotoxicity with either FLX or PER: i) attenuated adverse LV cavity remodelling; ii) decreased myofibrillar disarray and cardiomyocyte vacuolization; iii) lowered expression of HMGB1, a biomarker of necrosis and mitochondrially mediated ferroptosis; iv) lowered expression of Bnip-3, a biomarker of mitochondrial damage, apoptosis, and necrosis; and v) reduced inflammatory oxylipins in a chronic *in vivo* murine model.

5.1 Reversal of Adverse Cardiovascular Remodelling

Previous murine studies have demonstrated the efficacy of RAS antagonists in both the prevention and treatment of cardiotoxic changes induced by chemotherapy. In a study by Hiona *et al*., using a chronic 10-week rat model (n=24), they demonstrated that prophylactic administration of the ACEi Enalapril (10mg/kg/day) significantly attenuated loss of systolic function caused by DOX (25mg/kg). Additionally, prophylactic administration of Enalapril also fully prevented the increased mortality rate of 25% associated with the DOX treatment at the study endpoint.[216] In a study by Zhang *et al.*, using a chronic 4-week rat model (n=60), they demonstrated that concomitant administration of the ACEi Fosinopril (25mg/kg/day) prevented DOX (15mg/kg) induced elevations in LV filling pressure.[217] A more recent study by Akolkar *et al*. used a chronic

13-week mouse model (n=240) comparing three RAS inhibitors in the prevention of DOX (20mg/kg)+TRZ (20mg/kg) induced cardiotoxicity.[207] The study found that prophylactic administration of 50mg/kg/day of the DRI Aliskiren, 10mg/kg/day of the ARB Valsartan, or 3mg/kg/day of the ACEi PER partially attenuated adverse cardiovascular remodeling due to DOX+TRZ.[207]

Although a number of previous murine studies have focused on the prophylactic role of RAS antagonism in the prevention of chemotherapy mediated cardiotoxicity[207,216,217], these agents are also used in the treatment setting. In a study by Zhang et al, using a chronic 4-week rat model (n=60), they demonstrated that two weeks of treatment with the ACEi Fosinopril (25mg/kg/day), immediately following two weeks of DOX (15mg/kg) administration, reversed elevations in LV filling pressures.[217] In a study by Soga *et al.*, they used a chronic 10-week rat model (n=46) where Daunorubicin (9mg/kg) was administered over two weeks, followed by an additional four-week time period to allow for cardiomyopathy to become established.[218] Following this, the rats received placebo or the ARB Candesartan (5mg/kg/day) for an additional four weeks to evaluate for any recovery in LV systolic function.[218] Their study demonstrated that treatment with the ARB reduced Daunorubicin induced elevations in LV end-diastolic pressure and helped recover LV systolic dysfunction.[218]

In the clinical setting, both RAS antagonists and β-blockers are first line agents in the treatment of DOX+TRZ mediated cardiotoxicity.[107] In 2002, Jensen *et al*. conducted a prospective, blinded, long-term observational study evaluating 120 women with metastatic breast cancer who were treated with up to 1000mg/m^2 of EPR. In the subset of patients who developed heart failure (11%),

they were treated with either Enalapril (15mg/day) or Ramipril (10mg/day). In their study, Jensen *et al*. found that treatment with either ACEi increased the probability of LVEF recovery, that ACEi needed to be continued even after stabilization of cardiac function in order to maintain the recovered LVEF, and that the longer the delay of symptom onset following EPR treatment the more severe the cardiotoxicity.[219] In a 2010 prospective clinical trial, Cardinale *et al*. treated 201 cancer patients who developed anthracycline mediated cardiotoxicity (LVEF<45%) with increasing doses of Enalapril (up to 20mg/day) and Carvedilol (up to 50mg/day).[220] There was an improvement in LVEF in up to 55% of patients with the best indicator for success being a shorter time after anthracycline therapy to initiation of Enalapril and Carvedilol treatment.[220] Most recently, in 2019, a retrospective clinical study performed by Ohtani *et al*. was conducted in 350 patients who received serial TTE studies both before and after anthracycline therapy. The study focussed on the 52 patients (14.9%) that developed anthracycline induced cardiotoxicity and noted that the patients who promptly received higher doses of ACEi and β-blockers following early detection of cardiotoxicity were most likely to recover.[221]

As an alternative to pharmaceuticals, many have hypothesized that the nutraceutical FLX may have benefits in the context of chemothrapy mediated cardiotoxicity, following previous trials demonstrating the positive effect of dietary FLX in the treatment of atherosclerosis, arrythmias, hypertension, and sudden cardiac death.[168,185-205] Two previous murine studies from our laboratory examining the use of FLX in the context of CTRCD have shown that the ALA and SDG components of FLX are partially cardioprotective in a chronic *in vivo* female mouse model of DOX+TRX mediated cardiotoxicity.[168, 205] The latter study also showed that FLX offers equal cardioprotection to the current pharmacological standard of ACEi, when used in the prophylactic

setting.[205] Both of these pre-clinical studies demonstrated that prophylactic administration of FLX can partially prevent LV cavity dilatation and LV systolic dysfunction in mice treated with DOX+TRZ.[168,205] Whether the administration of FLX can ***reverse*** cardiac dysfunction after DOX+TRZ mediated cardiotoxicity requires further study.

In the current chronic *in vivo* study, we evaluated whether FLX, PER, or the combination can be used in the treatment of established DOX+TRZ mediated cardiotoxicity. On both a structural and a functional level, the groups receiving FLX, PER, and FLX+PER all demonstrated improved LV remodeling with an ~18% decrease in LVEDD and an ~40% increase in LVEF as compared to the DOX+TRZ group alone. While the LV cavity dimensions and LVEF values of the three experimental groups did not return to the baseline levels of the healthy controls, they still showed a significant improvement as compared to the mice treated with DOX+TRZ alone (Figures 9 & 10). This is the first study to demonstrate the equivalence of FLX as compared to the ACEi PER in the treatment of DOX+TRZ mediated cardiotoxicity in a murine model. The novel discovery that both FLX and PER successfully reverse DOX+TRZ induced heart failure raises the clinical query of whether nutraceuticals can be used in lieu of and/or in combination with pharmaceuticals for the management of CRTCD.

5.2 Histological Overview of Cardiotoxicity

Both DOX+TRZ have been associated with similar histopathological changes. Numerous studies have demonstrated that DOX alone causes a loss of cardiomyocyte integrity through mitochondrial swelling, vacuolization of the cytoplasm, dilatation of the sarcotubular system, formation of lysosomal bodies, loss of myofibril assembly, increased cardiomyocyte diameter, and increased

apoptosis and fibrosis.[149,168,190,207,222-225] It has also been shown that TRZ alone causes mitochondrial swelling, a decreased number of functional mitochondria, interstitial and perivascular infiltration of lymphocytes and macrophages, and cardiomyocyte apoptosis and necrosis.[168,207,226] Together, DOX+TRX have been shown to cause a loss of myofibril integrity and dilation in the perinuclear cisternae.[227] These pathological changes however, may be attenuated through concurrent treatment with pharmaceutical and nutraceutical agents.

Previous murine studies investigating the role of RAS antagonists in the prevention and treatment of cardiotoxic changes induced by chemotherapy have shown attenuation of histopathological changes in cardiac tissue. Hiona *et al*. evaluated a 10-week rat model (n=24) where prophylactic administration of 10mg/kg/day of Enalapril prevented myofibrillar degeneration (hematoxylin-eosin staining) caused by the administration of 25mg/kg of DOX.[216] Similarly, Soga *et al*. studied a 10-week recovery rat model (n=46) demonstrating that treatment with the ARB Candesartan (5mg/kg/day), following establishment of Daunorubicin (9mg/kg) mediated cardiotoxicity, resulted in lower levels of enlarged cardiomyocyte and fibrosis as compared to untreated mice using hematoxylin-eosin stained and Azan-Mallory stained light microscopy.[218] Additionally, Sakr *et al*. studied a 4-week rat model (n=156) where DOX (15 mg/kg) was administered for 2 weeks followed by treatment with the ARB Valsartan (10mg/kg/day) for an additional 2 weeks.[161] Using hematoxylin-eosin stained light microscopy, they demonstrated that the myocardial tissue from rats treated with Valsartan had less vacuolization, necrosis, and sarcoplasm granularity than the rats that received DOX alone.[161] Finally, in the study by Eekhoudt *et al*, we demonstrated that FLX and PER were equivalent in attenuating DOX+TRZ induced loss of cellular integrity, myofibril disarray, and vacuolization evaluated using electron microscopy. We also demonstrated

attenuation of increases in cardiac tissue biomarkers of inflammation, mitochondrial dysfunction, and apoptosis.[205]

In the current study, we performed histological analysis of tissue samples using both light microscopy with Masson's trichrome staining and electron microscopy. As compared to controls, mice treated with DOX+TRZ demonstrated significant myofibril degradation, vacuolization, and loss of sarcomere integrity (Figures 11 & 12). As a novel finding, the current study revealed significant improvement of DOX+TRZ induced myocyte damage in all three experimental groups using PER, FLX, or FLX+PER. The intermediate levels of cellular damage in the three experimental groups suggests that FLX and PER are both effective at partially attenuating DOX+TRZ mediated cardiotoxicity. When considering the light microscopy results, there does not appear to be any synergistic effect between FLX+PER, since there were no significant differences observed between the three experimental groups. However, when focusing on the electron microscopy results, the FLX+PER+DOX+TRZ group showed less damage as compared to the FLX+DOX+TRZ group, but not in comparison to the PER+DOX+TRZ group. This electron microscopy finding suggests possible synergy between FLX+PER. This discrepancy is likely explained by the inherent margin of error associated with the quantification of randomly selected tissue sections. It has been demonstrated that DOX induced cardiotoxicity is characterized by damage to random cardiomyocytes throughout the entire heart.[21] Combining the error associated with the random distribution of cell damage with the tissue section error likely accounts for the isolated appearance of synergy in the electron microscopy data. Alternatively, there could be slight synergy that is only detectible by electron microscopy because it is more sensitive than light

microscopy in detecting tissue damage; however, the finding is not corroborated by our echocardiographic or biochemical data.

The similarities in cardiomyocyte ultrastructure changes between the previous prophylactic studies[205,216] and the current recovery study may be explained by the reversibility of some types of cellular damage. While the delayed initiation of FLX+PER administration in the current study could allow for an accumulation of damage, it is possible that the early damage is an accumulation of reversible changes. Therefore, once the antioxidant and anti-inflammatory effects of FLX or PER create a microenvironment surrounding each cell that is more favourable for healing, some cells can repair their damage instead of completing a cell death pathway and becoming fibrotic, resulting in similar histological outcomes. This is supported by the similar results observed between the current recovery study and two previous recovery studies showing lower levels of tissue damage and fibrosis in RAS inhibitor treated groups as compared to treatment with anthracyclines alone.[161,218] However, to the extent of our knowledge, the current study is the first murine model to examine ACEi treatment for DOX+TRZ induced cardiotoxicity in the recovery setting, making our discoveries about both PER and FLX novel contributions to the field of Cardio-Oncology.

5.3 Mechanistic Pathways of Cardiotoxicity

In our study, mechanistic pathways elucidated through protein and lipid analyses show: i) attenuation of mitochondrial induced ferroptosis, apoptosis, and necrosis in cardiomyocytes; and ii) decreased circulating plasma markers of inflammation with FLX+PER treatment as compared to DOX+TRZ alone.[73,123-127,168,228-230] DOX+TRZ mediated cardiotoxicity is characterized by

randomly distributed cardiomyocyte death leading to functional decline.[21] Ferroptosis, apoptosis, and necrosis can all contribute to this cell death pattern. Considering the functional and cellular investigations, it is clear that FLX does not fully attenuate DOX+TRX mediated cardiotoxicity. However, our current biomarker results suggest that FLX may fully mitigate the effects of oxidative stress leaving other damage mechanisms responsible for the remaining functional decline.

5.3.1 Mitochondrial Induced Ferroptosis

Through its multimodal cardiotoxic mechanisms, DOX activates multiple cell death pathways. One of the primary mechanisms of DOX induced cardiotoxicity is oxidative damage of cardiomyocytes.[21,49,51,97,98,120,168,205,212] In the presence of iron, oxidative stress causes ferrous and ferric cations to accumulate in the mitochondria which reduces the mitochondrial membrane potential.[124] Without the necessary membrane potential, cardiomyocyte mitochondria cannot maintain the energy production required for repetitive myocyte contractions. This iron induced energy crisis then triggers a pre-programmed ferroptotic cell death pathway. Previous studies have shown that ferroptosis is associated with HMGB1 release, particularly in models of DOX induced cardiotoxicity.[124] To elucidate the roles of various cell death pathways in DOX induced cardiotoxicity, two pre-clinical studies treated mice or rats with inhibitors of ferroptosis, necroptosis, apoptosis, or autophagy prior to the induction of DOX mediated cardiomyopathy (20mg/kg DOX). Both studies found that only inhibition of ferroptosis had a cardioprotective effect that reduced overall mortality due to DOX.[124,228]

In the current study, HMGB1 expression was elevated in the DOX+TRZ group, demonstrating that oxidative stress induced ferroptosis. However, HMB1 expression was attenuated to the level of healthy controls in mice treated with FLX, PER, or FLX+PER. It is likely that DOX+TRZ induced iron ion accumulation in the mitochondria leading the cardiomyocytes towards ferroptosis. Our results suggest that PER and the antioxidant properties of FLX stopped the progression of ferroptosis in the three experimental groups allowing cardiomyocytes to recover instead of completing the cell death pathway. While the increased HMGB1 levels in the DOX+TRZ group aligns with the established literature [123-126,228], the attenuation of HMGB1 by FLX and PER is a novel discovery that adds to the established evidence that antioxidants can temper the cardiotoxic side effects of DOX by reducing cell death.

Despite the complete attenuation of changes in HMGB1 expression (Figure 13), total cardiac recovery was not observed in functional and cellular investigations reflective of the multivariable nature of DOX+TRX mediated cardiotoxicity. In addition to indicating ferroptosis, HMGB1 acts as a marker of necrosis, as it is passively released from necrotic cells. HMGB1 can also trigger inflammation in neighbouring cells which contributes to DOX induced cardiomyocyte apoptosis, cell damage, and cardiac fibrosis.[123] While HMGB1 has increased expression in murine models of DOX+TRZ induced cardiotoxicity, it remains unclear if it is most strongly associated with necrosis, inflammation, ferroptosis, or apoptosis in the cardiotoxic setting. Further studies are warranted to elucidate the source of HMGB1 in DOX+TRZ mediated cardiotoxicity and the relative contributions of the various cell death pathways to the development of CTRCD.

5.3.2 Mitochondrial Induced Apoptosis & Necrosis

Through exposure to DOX, cardiomyocytes endure oxidative damage as another mechanism of cardiotoxicity. Oxidative stress upregulates the production of Bnip-3, which then damages the mitochondrial membranes, compromising the cell's ability to produce the energy required for repetitive myocyte contractions.[73] The resulting energy crisis triggers apoptotic and necrotic cell death pathways. As the number of apoptotic and necrotic cardiomyocytes increases, the heart exhibits pathological structural changes and functional decline.

The importance of Bnip-3 in DOX mediated cardiotoxicity was elucidated by a previous study that assessed the direct link between Bnip-3 expression and DOX induced damage using three different methodological approaches.[73] It has been established that DOX induces mitochondrial dysfunction, that the mitochondria act as a signalling platform for apoptosis and necrosis, and that Bnip-3 can trigger mitochondrial perturbations.[73] These perturbations include mitochondrial permeability transition pore opening, loss of mitochondrial membrane potential, and necrotic cell death, so Dhingra *et al*. hypothesized that Bnip-3 may be the link between DOX and mitochondrial damage. First, the authors used an *in vivo* mouse model to evaluate the characteristics of 20 mg/kg DOX induced mitochondrial damage and found that mice receiving DOX had increased Bnip-3 expression levels. Second, they used an *in vitro* model and found a dose-dependent relationship between DOX and Bnip-3 increases in ventricular myocytes. Both *in vitro* and *in vivo* methods found that the mitochondrial injury induced by DOX was consistent with the mitochondrial injury induced by Bnip-3. The *in vitro* model also showed that inhibition of Bnip-3 using small hairpin ribonucleic acid or a carboxyl terminal transmembrane domain mutant of Bnip-3 (negative inhibitor of Bnip-3) restored mitochondrial respiration and cell viability.[73] Additionally, Dhingra

et al. used an *in vivo* mouse model demonstrating that Bnip-3 knockout mice were resistant to misaligned sarcomeres, disrupted mitochondrial cristae, and vacuolization typically induced by DOX (20mg/kg).[73] Finally, a study by Asselin *et al*. demonstrated that Bnip-3 is upregulated by nearly 2-fold after treatment with DOX+TRZ.[168] Prophylactic administration of FLX prevented an increase in Bnip-3 expression in this murine model of DOX+TRZ mediated cardiotoxicity.[168] Overall, DOX exerts its mitochondrial damage *via* the upregulation of Bnip-3 which may be downregulated by the prophylactic administration of FLX.[73,168]

In the present study, we demonstrated that Bnip-3 expression was increased by 2.3-fold in mice treated with DOX+TRZ. However, treatment with either FLX, PER, or the combination of FLX+PER attenuated the Bnip-3 levels back to the level of the healthy controls. These effects are likely due to the antioxidant properties of FLX and PER.[73,123-126,158,168,228] In the DOX+TRZ group, oxidative stress upregulates Bnip-3, compromising the integrity of the mitochondria, causing cardiomyocytes to enter an energy crisis and initiate apoptotic and necrotic cell death pathways, ultimately leading to functional changes in the heart. In the three experimental groups, however, the antioxidant properties of PER and FLX attenuated the oxidative stress, thus protecting the mitochondria and preventing apoptosis and necrosis in the cardiomyocytes. Despite the complete attenuation of changes in Bnip-3 expression (Figure 14), complete reversal of cardiac damage was not observed in functional and cellular investigations, reflective of the multivariable nature of DOX+TRX mediated cardiotoxicity.

The current study presents novel evidence that FLX and PER attenuate Bnip-3 mediated damage and adds to the existing evidence that the mitochondria play a vital role in CTRCD. It is therefore

feasible to consider that the apparent targeting of cardiomyocytes for DOX induced damage results from the high concentration of mitochondria present. As expected, based on the literature, DOX+TRZ increases expression of both HMGB1 and Bnip-3, indicating increased mitochondrial damage, apoptosis, necrosis, and ferroptosis. [73,123-126,168,228] As these are all induced by oxidative stress, and were all downregulated by FLX, the current evidence supports existing studies claiming that oxidative stress is the primary mediator of DOX+TRZ mediated cardiotoxicity. However, it remains unclear what proportions of DOX+TRZ mediated cardiotoxicity are attributable to oxidative stress and mitochondrial dysfunction alone. The relative distributions of cell death attributable to ferroptosis, apoptosis, and necrosis respectively also remains unclear. Further studies are warranted to elucidate the relative contributions of mitochondrial damage and various associated cell death pathways to the development of CTRCD.

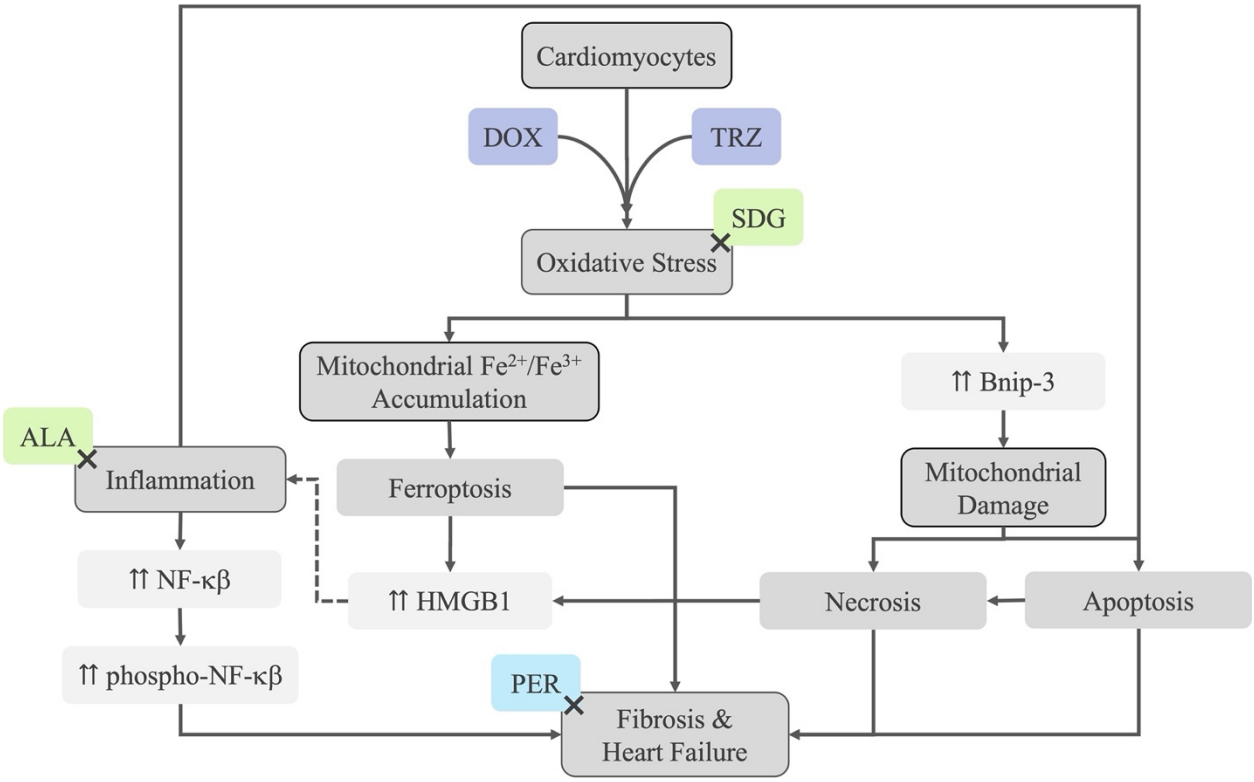

Figure 17. Mechanisms of FLX in the treatment of DOX+TRZ mediated cardiotoxicity. In the current study, FLX inhibited DOX+TRZ mediated oxidative stress in the mitochondria, attenuating elevations of Bnip-3 and HMGB1, suggesting the inhibition of cardiomyocyte ferroptosis, apoptosis, and necrosis, thus contributing to cardiac recovery.

ALA, Alpha-linoleic acid; Bnip-3, Bcl-2 interacting protein 3; DOX, Doxorubicin; HMGB1, High mobility group box 1 protein; NF-κβ, Nuclear factor kappa beta; PER, Perindopril; SDG, Secoisolariciresinol diglucoside; TRZ, Trastuzumab.

5.3.3 Inflammatory Changes in Cardiotoxicity

In addition to inducing changes in protein expression, DOX+TRZ can elicit changes in lipid metabolism.[229] Generated through PUFA oxidation, oxylipins are the active metabolites of PUFAs and are responsible for mediating the physiological effects of PUFAs throughout the body.[127] Oxylipins can be produced from n-3 or n-6 PUFAs *via* the COX, LOX, and CYP pathways, with n-6 PUFA derived oxylipins generally presenting more inflammatory, vasoconstrictory, and proliferative effects than n-3 PUFA derived oxylipins.[127] The CYP ω-hydroxylase pathway

produces HETEs from arachidonic acid (n-6). The enzymes producing CYP ω-hydroxylase derived HETEs are highly expressed in cardiomyocytes.[230] These oxylipins mediate their renal, vascular, and cardiac effects *via* receptors, cross-reactions with other oxylipin receptors, or modulating intracellular transcription factors and ion channels.[127] While some oxylipin effects have been elucidated, many remain unknown and and/or contradictory. For example, within the CYP ω-hydroxylase pathway, 16-, 18-, and 19-HETE, and 20-COOH-AA can promote vasodilation while 20-HETE has shown hypertensive, pro-inflammatory, and pro-apoptotic effects.[127,229,230] Some studies have found a link between 20-HETE and activation of the RAS in hypertension.[229] 20-HETE synthesis has also been shown to be upregulated in models of DOX induced cardiotoxicity.[229] Studies in cardiovascular disease have shown that inhibition of 20-HETE production can decrease hypertension, MI size, and heart failure by exerting antioxidant, anti-inflammatory, and anti-apoptotic effects. Additionally, in a model of diabetic cardiomyopathy, increased circulating levels of 20-HETEs may have induced the increased myocardial injury.[230] 20-HETEs were shown to upregulate a pro-fibrotic cardiac signaling pathway (TGF-β pathway) that is directly activated by ROS and plays a major role in cardiac fibrosis. While the data suggests that increased circulating and LV 20-HETEs contribute to diabetic cardiomyopathy[230], the exact mechanism remains to be elucidated.

In the context of cancer, 20-HETE has been implicated in the proliferation of tumor cells *via* the upregulation growth factors and angiogenesis.[229] Inhibition of the 20-HETE pathway has reduced tumor size in *in vivo* models of brain, kidney, and breast cancers.[229] The 20-HETE pathway is therefore a potential target for anti-cancer therapies.[229]

In our current study, in mice treated with DOX+TRZ, there was increased plasma concentration of 18-HETE and 20-COOH-AA as compared to healthy control mice (Figures 15 & 16). Elevations in these oxylipins were significantly downregulated in mice treated with FLX, PER, or FLX+PER. While the functions of these two significant oxylipins are not well characterized, they are generated by a metabolic pathway that has been associated with vascular tone, oxidative stress, apoptosis, inflammation, cardiac fibrosis, angiogenesis, and tumor proliferation.[127,229,230]

While the oxylipin concentrations found in the current study align with the conclusion that FLX partially attenuates the cardiotoxic mechanisms of DOX+TRZ, there are multiple ways the oxylipin data could contribute to the previously discussed results. One possibility is that the attenuation of elevations in 18-HETE and 20-COOH-AA plasma levels further confirm that FLX and PER reduce: i) oxidative stress induced apoptosis mediated *via* the mitochondria; and ii) HMGB1 induced inflammation leading to cardiac fibrosis. Alternatively, these results could be indicative of an independent cardiotoxic mechanism involving oxidative stress and inflammation leading to apoptosis and cardiac fibrosis. A third possibility is that there is partial overlap between the mechanisms. Specifically, if oxidative stress induced apoptosis causes upregulation of 18-HETE and 20-COOH-AA, it could lead to HMGB1 independent inflammation leading to cardiac fibrosis. Irrespective of the specific mechanism, the antioxidant and anti-inflammatory properties of SDG and ALA in FLX appear to attenuate the cardiotoxic effects of elevated CYP ω-hydroxylase oxylipins.

Additionally, considering the increased angiogenesis and tumor proliferation associated with CYP ω-hydroxylase oxylipins, FLX may exhibit anti-cancer effects by attenuating this pathway. Further

studies are warranted to elucidate the specific roles of 18-HETE and 20-COOH-AA in DOX+TRZ induced cardiotoxicity and the mechanisms through which FLX and PER inhibit the pathological effects of these oxylipins. Considering the possible anti-cancer effects of FLX in addition to the cardioprotective effects, it is especially important to explore these metabolic pathways in the context of Cardio-Oncology.

5.4 Limitations

There are a number of limitations associated with the current study. First, although DOX+TRX were administered concurrently to create a robust model of chemotherapy mediated cardiotoxicity[168,205], these anti-cancer agents are typically administered sequentially in the clinical setting. Further research is warranted to characterize DOX+TRZ cardiotoxicity and possible treatment effects of FLX+PER by administering the anti-cancer agents sequentially. Second, our chronic *in vivo* murine model for the current study consisted entirely of female mice. As breast cancer, and the associated use of cardiotoxic anti-cancer therapies, is not exclusive to females, further research is warranted to evaluate the cardiovascular treatment potential of FLX+PER in male models. Finally, the current study did not evaluate whether FLX or PER may impact the anti-neoplastic properties of DOX+TRZ. Further research is warranted to ensure that FLX+PER consumption does not negatively impact the anti-cancer effects of DOX+TRZ.

5.5 Future Directions & Clinical Implications

In order to fully translate the role of FLX+PER in the clinical treatment of DOX+TRX mediated cardiotoxicity, further investigations are warranted. First, both *in vitro* and *in vivo* pre-clinical studies are recommended to confirm that treatment with FLX+PER will not compromise the anti-

neoplastic properties of DOX+TRZ. Second, a double-blinded, placebo-controlled, randomized clinical trial is warranted to investigate the clinical effectiveness of FLX as a treatment agent for DOX+TRZ mediated cardiotoxicity in the breast cancer setting. Finally, further clinical studies are warranted to compare the efficacy and feasibility of dietary FLX used at different timepoints, either as prophylactic, concurrent, or subsequent treatment, in the context of chemotherapy mediated cardiotoxicity.

The current study has tremendous clinical implications. The current standard of practice with cancer patients is to hold vital anti-cancer therapies and initiate heart failure medications, including ACE inhibitors, once overt cardiotoxicity has developed. This approach not only provides an opportunity for their malignancy to spread, but it can also generate new adverse side effects associated with the heart failure medications including light-headedness and fatigue from ACEi and β-blocker use.[168,207,231] While corroborating clinical trials are essential, our novel pre-clinical findings have shown that consumption of the nutraceutical FLX is comparable to the current standard of care pharmaceutical agent ACEi in the treatment of DOX+TRZ mediated cardiotoxicity. As such, consumption of FLX may prove to be a favourable and feasible alternative to PER due to its improved tolerability and additional health benefits.[202,232]

Chapter 6: Conclusions

Our novel study has shown that the administration of FLX or PER was able to treat the cardiotoxic effects of DOX+TRZ in a chronic *in vivo* female murine model. Treatment with the combination of FLX+PER, however, was not synergistic in attenuating the cardiotoxicity associated with DOX+TRZ.

Based on the results of the current study, we can conclude that FLX was equivalent to PER at attenuating changes in adverse cardiovascular remodeling, reducing histopathological changes in cardiomyocyte ultrastructure, reducing biomarkers of mitochondrial induced cell death, and reducing circulating levels of arachidonic acid-derived CYP ω-hydroxylase oxylipins.

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